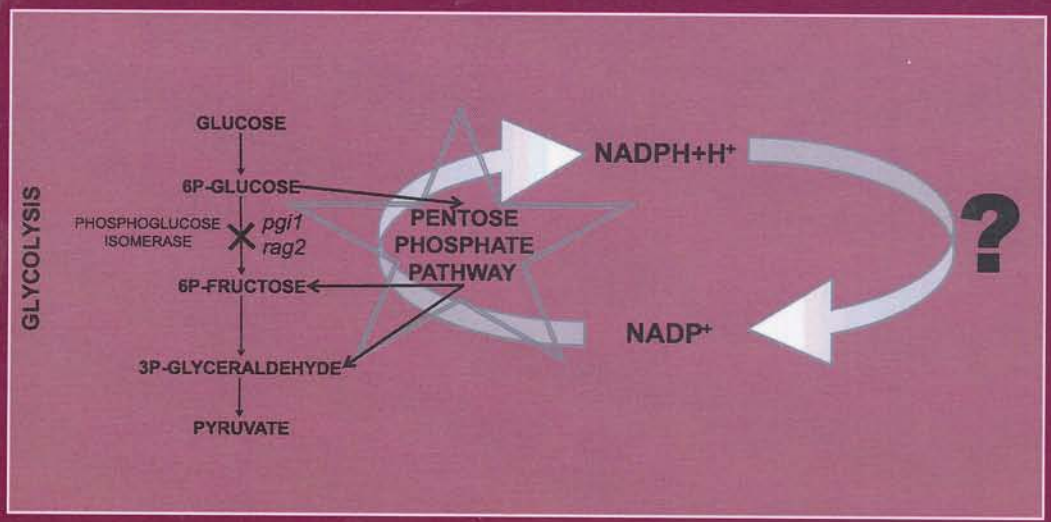

CYTOSOLIC NADPH REOXIDATION IN *Kluyveromyces lactis*

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Memoria para aspirar al grado de
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presentada por

Nuria Tarrío Yáñez

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El presente trabajo, **Cytosolic NADPH reoxidation in *Kluyveromyces lactis***, presentado por Nuria Tarrío Yáñez para aspirar al grado de Doctor en Biología, ha sido realizado bajo mi dirección en el Departamento de Biología Celular y Molecular de la Universidad de A Coruña.

Revisado el texto, estoy conforme con su presentación para ser juzgado.

A Coruña, 20 de enero de 2006

V°B°

Dra. María Isabel González Siso

Profesora de Bioquímica y Biología Molecular.

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A Sony
y a mis padres

Deprende, meu velliño,
a cencia ben amada,
que saibamente insina
tan rica misturanza,
si queres ser sabido
en cousas tan estrañas,
pois antre tantas novas
as costumiñas rancias...
Non che digo nada...
Pero ¡vaia!

Rosalía de Castro
Cantares Gallegos (1863)

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INTRODUCTION

Kluyveromyces lactis versus *Saccharomyces cerevisiae*

Kluyveromyces lactis, originally called *Saccharomyces lactis*, has been studied since the beginning of the 1960s. *K. lactis* has a genome of 12 million base pairs, slightly smaller than that of *S. cerevisiae*, but has only six chromosomes while *S. cerevisiae* has sixteen. *Kluyveromyces* and *Saccharomyces* genera originate from an ancient common ancestor (Kellis *et al.* 2004). The divergence of *Saccharomyces* from *Kluyveromyces* occurred before the proposed whole-genome duplication of *S. cerevisiae*. After tetraploidy, most of the genes were deleted but a small fraction of the genes were retained in duplicate and gene order was rearranged by many reciprocal translocations between chromosomes. Protein pairs derived from this duplication event, which appear in *S. cerevisiae* and not in *K. lactis*, make up 13% of all yeast proteins (Wolfe and Shields, 1997)

The *K. lactis* development as a useful yeast for investigation has been facilitated because a lot of molecular genetic techniques that had been developed from the conventional yeast *S. cerevisiae* are useful for *K. lactis* as well, and because its evolutionary distance with *S. cerevisiae* makes possible the functional domain comparison from homologous genes. The elaboration of specific vectors, based on either the 2 μ-like plasmid pKD1, the *K. lactis* autonomously replicating sequences (KARs) or the *K. lactis* centromeric sequences (KlCENs) has considerably contributed to this development (Schaffrath and Breunig, 2000).

K. lactis became an important alternative to *S. cerevisiae* in industrial applications, because of its advantages related to the ability to grow in a wider variety of inexpensive carbon sources (Barnett *et al.*, 1990), the higher efficiency shown in the heterologous production of secreted proteins (van den Berg *et al.*, 1990), the different characteristics of the respiro-fermentative metabolism (González Siso *et al.*, 2000) and the Killer phenotype which have some *K. lactis* strains conferred by a pair of lineal cytoplasmic DNA plasmids (Stark *et al.*, 1990; Wésolowski-Louvel *et al.*, 1996).

Some important physiological differences between *K. lactis* and *S. cerevisiae* are:

a) Lactose metabolism

Lactose is the most outstanding of the carbon sources that are catabolized by *K. lactis* but not by *S. cerevisiae* (Wésolowski-Louvel *et al.*, 1996). The *LAC4* gene encodes for β-galactosidase and the *LAC12* gene encodes for lactose permease, both genes give *K. lactis* the ability to grow in lactose. The transference of these genes to *S. cerevisiae* promotes the growth of *S. cerevisiae* in lactose media (Sreekrishna *et al.*, 1985; Breuning *et al.*, 2000, Becerra *et al.*, 2003). This is of biotechnological interest

because lactose is present in cheese whey, a waste subproduct, and *K. lactis* is used as a source of β-galactosidase (Becerra *et al.*, 2003).

b) Ethanol metabolism

K. lactis is not a good ethanol producer in comparison with *S. cerevisiae*. Four alcohol dehydrogenase genes (*KlADH1, KlADH2, KlADH3* and *KlADH4*) have been described in *K. lactis*. The proteins coded by *KlADH1* and *KlADH2* are cytosolic enzymes that in their regulation resemble the *S. cerevisiae* gene *ScADH1*, whereas the *KlADH3* and *KlADH4* encoded proteins are mitochondrial. *KlADH4* is induced by ethanol and insensitive to glucose repression, a mode of regulation not found in *S. cerevisiae*, whereas *KlADH3* is repressed by ethanol and by high concentrations of glucose (Saliola and Falcone, 1995, Breuning *et al.*, 2000).

c) Hexoses transporters genes

S. cerevisiae genome has a subfamily of 18 genes which encode for hexose transporters (*HXT*) and it has been established that 17 of them can transport glucose (Reifenberger *et al.*, 1995; Boles and Hollenberg, 1997; Wieczorke *et al.*, 1999). So, mutants in an individual gene *HXT* do not show a clear mutant phenotype. In contrast, *K. lactis* has only the gene *HGT1* encoding a high-affinity glucose transporter (Billard *et al.* 1996) and the gene *RAG1* which encodes a low affinity permease (Goffrini *et al.* 1990). *RAG1* is a chimeric gene that has arisen by recombination between two genes, *KHT1* and *KHT2*, which are found in tandem in some strains instead of *RAG1* (Milkowski *et al.*, 2001).

d) Respiro-fermentative metabolism

K. lactis and *S. cerevisiae* are facultative aerobic yeasts that can metabolize the glucose by both oxidative and fermentative pathways, but *K. lactis* metabolism is preferably respiratory whereas *S. cerevisiae* metabolism is basically fermentative (Gancedo y Serrano, 1989)

In *S. cerevisiae* the use of oxygen in glucose-limited fully-oxidative continuous cultures increases linearly with the dilution rate to arrive to a point from where oxygen consumption rate remains constant and ethanol production increases, this means that the respiratory capacity is saturated and the carbon flux is partially directed to the ethanol production (Alexander and Jeffries, 1990). In *K. lactis*, this saturation of the respiratory capacity does not occur (González Siso *et al.*, 2000). *K. lactis* is classified as Crabtree-negative yeast whereas *S. cerevisiae* is Crabtree-positive yeast (Crabtree, 1929; de Deken, 1966). Mulder et al (1995) propose that this classification of *K. lactis* concerns only the short-term Crabtree effect because *K. lactis* produces ethanol during steady-state growth on glucose under aerobic conditions. In *K. lactis* respiratory and fermentative metabolism can work in parallel. The mechanisms that determine the *K.*

lactis unlimited respiratory capacities are not fully understood, but there is experimental evidence that they operate at different control levels. An outline of some of these mechanisms follows.

There are high expression levels in the *K. lactis* genes that code for mitochondrial proteins. The quantitative determination of *CYC1* mRNA levels in *K. lactis* and *S. cerevisiae* wild type strains have demonstrated a higher expression of *KlCYC1* than *ScCYC1* (Freire Picos *et al.*, 1994). High expression levels also have been found in *KlHEM1* (González Domínguez *et al.*, 1997) encoding the first enzyme of biosynthesis of heme. Heme controls the expression of some genes related to the respiratory function in *S. cerevisiae* (Zitomer and Lowry, 1992) which also are expressed at high levels in *K. lactis*

Catabolic repression of genes related to respiratory functions in *K. lactis* has been shown to be slight compared to *S. cerevisiae* or not to occur at all (Mulder *et al.*, 1995). Moreover, the glycolytic pathway in *K. lactis* is not repressed by glucose and this yeast does not have many duplicated glycolytic genes as *S. cerevisiae* (Blaisonneau *et al.*, 1997). The proposed genome duplication may have facilitated the evolution of anaerobic fermentation in *Saccharomyces*; for example, the duplicated genes include several pairs that are regulated differently under aerobic and anaerobic conditions (*CYC1* and *CYC7*, *COX5A* and *COX5B*), as well as several genes including sugar transporters (Wolfe and Shields, 1997).

e) Pentose phosphate pathway

Pentose phosphate pathway is an important metabolic route implicated in production of NADPH for biosynthetic reactions, 5-phosphate ribose for nucleic acids and nucleotidic cofactors biosynthesis and 4-phosphate eritrose for aromatic amino acids biosynthesis. The glucose metabolism is divided between glycolysis and pentose phosphate pathway at the level of 6-phosphate glucose. It was reported that the nitrogen source in the culture influences the amount of glucose driven to pentose phosphate pathway (Lagunas and Gancedo, 1973)

The transaldolase enzyme catalyzes the reversible reaction from 4-phosphate eritrose and 6-phosphate fructose to 3-phosphate glyceraldehyde and 7-phosphate sedoheptulose. The specify activity of this enzyme in *K. lactis* is 5-fold higher than *S. cerevisiae* transaldolase (Jacoby *et al.*, 1993) Moreover, *K. lactis* mutants lacking this activity encoded by *KlTAL1* gene, have been tools to elucidate an important difference between these yeasts, *K. lactis* can use glucose only by the pentose phosphate pathway while *S. cerevisiae* is unable (Jacoby *et al.*, 1993)

The importance of the pentose phosphate pathway in *K. lactis* is also supported by a study of metabolic flux analysis in 14 hemiascomycetous yeasts. *Kluyveromyces lactis* is among the species with the highest relative flux of glucose through the pentose phosphate pathway whereas *S. cerevisiae* is among those with the lowest. Moreover, pentose phosphate pathway fluxes correlate with Krebs cycle fluxes and with the yields of biomass (Blank *et al.*, 2005)

THE *rag2* MUTANT AS A TOOL TO STUDY CYTOSOLIC NADPH REOXIDATION IN *K. lactis*

Some strains of *K. lactis* are able to grow in glucose in the presence of antimycin A which is an inhibitor of the mitochondrial respiratory chain. In this way, the Rag^+ phenotype was defined as Resistance to Antimycin in Glucose and constituted an important tool for the study about the genes involved in the glucose metabolism in *K. lactis* (Goffrini *et al.*, 1989)

The *RAG2* gene encodes for the enzyme phosphoglucose isomerase (Wésolowski-Louvel *et al.*, 1988; Chen *et al.*, 1992) which catalyzes the second reaction in the glycolysis. The deficit of this enzyme does not allow the normal glucose metabolism via glycolysis because 6-phosphate glucose cannot be converted into 6-phosphate fructose, therefore the 6-phosphate glucose is diverted to the pentose phosphate pathway, which generates an increased amount of NADPH reducing power (Fig. 1).

The *K. lactis rag2* mutant shows a different phenotype than the *S. cerevisiae* mutant in the same enzyme (*pgi1*) in relation to its growth in media with glucose as sole carbon source. Whereas *S. cerevisiae* mutant cannot grow in these media (Ciriacy and Breitenbach, 1979; Aguilera, 1987) the *K. lactis* mutant can (Goffrini *et al.*, 1991; González Siso *et al.*, 1996a). In *K. lactis,* when the deletion in the phosphoglucose isomerase gene was joined with the deletion in the pentose phosphate pathway transaldolase gene, the result was that the double mutant was not able to grow in glucose; this effect is also shown in the triple mutant devoid in the two phosphofructokinase genes and transaldolase gene (Jacoby *et al.*, 1993). This confirmed that *K. lactis* uses the pentose phosphate pathway when the glycolysis is blocked.

The growth in glucose of the *S. cerevisiae pgi1* mutant has been restored either by inducing a transhydrogenase system by NAD-dependent glutamate dehydrogenase gene overexpression or by submitting the cells to oxidative stress, for instance, by addition of hydrogen peroxide (Boles *et al.*, 1993). Therefore, the inability of *S.*

cerevisiae pgi1 mutant to grow in glucose has been attributed to its inability to directly reoxidize the NADPH generated in excess by the pentose phosphate pathway, this route being blocked by the lack of $NADP^+$ available (Boles *et al.*, 1993). Thus, more efficient mechanisms of NADPH reoxidation in *K. lactis* than in *S. cerevisiae* have been proposed as the key to the differences between the phenotype of this mutant and the *K. lactis rag2* mutant (González Siso *et al.*, 1996a).

The growth in glucose of the *K. lactis rag2* mutant is avoided by the mitochondrial respiratory inhibitor antimycin A (Goffrini *et al.*, 1991). This suggested that in *K. lactis* the cytosolic NADPH generated by the pentose phosphate pathway can be efficiently reoxidized, either directly or indirectly by a shuttle mechanism, by the action of the respiratory chain (González Siso *et al.*, 1996a)

Previous studies in our laboratory suggested the hypothesis that *K. lactis* has a mitochondrial external alternative dehydrogenase with the capacity to oxidize NADPH and transfer electrons to ubiquinone (González Siso *et al.*, 1996a). Later, this hypothesis was supported by the discovery of two external alternative dehydrogenases in *S. cerevisiae* mitochondria which are NADH-specific (Luttik *et al.*, 1998; Small and McAlister-Henn, 1998) and by the demonstration that *K. lactis* isolated mitochondria can oxidize exogenously added NADPH, this activity being induced in a *rag2* compared to a wild type strain (Overkamp *et al.*, 2002). However, other additional mechanisms may be implied also in the different efficacy in NADPH oxidation between *K. lactis* and *S. cerevisiae* (Fig. 1).

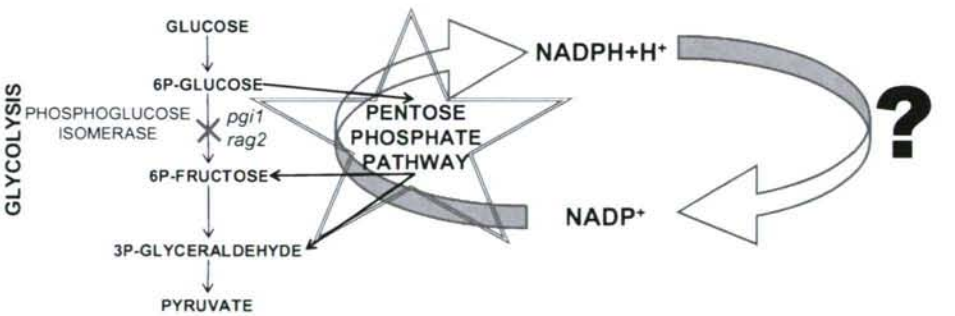

Figure 1: Scheme about NADPH generation by the pentose phosphate pathway in the phosphoglucose isomerase mutants.

NADPH TURNOVER IN YEASTS

In yeast's metabolism, the pyridine nucleotide cofactors play an important role. The NADH, nicotinamide adenine dinucleotide reduced form, is mainly used in dissimilatory metabolism, whereas NADPH, nicotinamide adenine dinucleotide phosphate reduced form, is usually used for assimilatory reactions (van Dijken and Scheffers, 1986). In the yeasts studied hitherto, NADH and NADPH are not directly interconvertible because of the lack of transhydrogenase activity (Bruinenberg *et al.*, 1985a; Camougrand *et al.*, 1988; Nissen *et al.*, 2001).

The cytosolic NADPH is produced in *S. cerevisiae* by the oxidative part of the pentose phosphate pathway, the $NADP^+$-specific isocitrate dehydrogenase and the aldehyde dehydrogenase (Ald6p) (Bruinenberg *et al.*, 1983; Minard *et al.*, 1998; Grabowska and Chelstowska, 2003). Despite the fact that the three enzymes produce NADPH, their role in the cell is different; Minard and McAlister-Henn (2005) demonstrated that glucose 6-phosphate dehydrogenase is the main source of NADPH when the cells are cultured in glucose, whereas the activity of $NADP^+$-specific isocitrate dehydrogenase increase when the cells are cultured in non-fermentable carbon source (lactate).

NADPH reoxidation mechanisms in the cell are necessary, because $NADP^+$ is limited and not accumulated by the cell. *S. cerevisiae*, different to other yeasts, cannot oxidize NADPH directly in the respiratory chain, NADPH reoxidation in this yeast takes place in the cytoplasm (Bruinenberg *et al.*, 1983; Alberts *et al.*, 1998). In this yeast the NADPH role is limited in the glucose fermentation since its cytosolic alcohol dehydrogenases are NADH-dependent (Ciriacy, 1997).

There are several possible pathways for NADPH reoxidation in yeasts; in this work we have studied the possible operation in *K. lactis* of the following: macromolecular biosynthesis, transhydrogenase cycles, mitochondrial external alternative dehydrogenases and oxidative stress response.

BIOSYNTHETIC ROUTES

In the majority of cases during the biosynthesis, the precursors are more oxidized than the final products, which mean that in addition to ATP, reducing power is also necessary. NADPH is the electron donor used, almost exclusively, by biosynthesis. The additional phosphate group is an identification signal by the correspondent biosynthetic enzymes.

The NADPH is used in fungi for lipids, sterols, amino acids, purines and deoxiribonucleotides biosynthesis, xilose and arabinose catabolism to sugar alcohols and for reducing nitric oxide, among other routes (Verho *et al.*, 2002).

TRANSHYDROGENASE CYCLES

Transhydrogenase cycles have been described in yeasts which may help to reoxidize the cytoplasmic NADPH. They are constituted by pairs of isoenzymes which are able to catalyze the same reversible reaction but in the opposite direction and using different coenzymes, thus transforming NADPH and NAD into NADH and $NADP^+$. These cycles can be found in a exclusive cellular compartment (cytoplasm) as the cycle generated in *S. cerevisiae* by the NAD- and NADPH-dependent glutamate dehydrogenases (Boles *et al.*, 1993) or between two cellular compartments (cytoplasm and mitochondria). This type is generated in *K. lactis* by one cytoplasmic acetaldehyde reductase using NADPH and one mitochondrial alcohol dehydrogenase encoded by the *ADH3* gene (Overkamp *et al.*, 2002)

Boles *et al.* (1993) observed that the phenotype of growth on glucose of the *pgi1* mutant in *S. cerevisiae* was restored by *GDH2* gene overexpression. This gene encodes for the NAD-dependent glutamate dehydrogenase enzyme (Altschul *et al.*, 1990) which catalizes the reaction: glutamate + NAD = 2-oxoglutarate $+NH_4^+$ +NADH. The overexpression of this gene decreases the cellular levels of glutamate and these low levels active another enzyme implied in the process, which is the NADPH-dependent glutamate dehydrogenase encoded by *GDH1* (Moye *et al.*, 1985) or *GDH3* (Bussey *et al.*, 1995) and that catalyzes the inverse reaction: 2-oxoglutarate + NH_4^+ + NAPDH = glutamate + $NADP^+$.

This transhydrogenase cycle (Fig. 2) restores the growth on glucose of the *pgi1* mutant due to the fact that the inefficient reoxidation of cytoplasmic NADPH is the single reason that makes *pgi1* mutant unable to grow in media with glucose as sole carbon source (Boles *et al.*, 1993). Thus, the pentose phosphate pathway is blocked by the lack of $NADP^+$, which is necessary as cofactor of two enzymes involved in this route.

This transhydrogenase cycle might operate in *K. lactis* so that the *rag2* mutant can reoxidize NADPH and grow in glucose. However, the glutamate metabolism is different between *S. cerevisiae* and *K. lactis*, *S. cerevisiae* being the single organism hitherto reported with three pathways for glutamate biosynthesis such as Gdh1p, Gdh3p and GOGAT (glutamate synthase) (Avendaño *et al.*, 1997). *S. cerevisiae* strains devoid of Gdh1p showed two fold slower doubling time than the wild type

growing on minimal media supplemented with ammonia as the sole carbon source (Folch *et al.*, 1989). These data suggested that Gdh1p constitutes the predominant pathway for glutamate biosynthesis (Avendaño *et al.*, 1997). In fact, it has been reported that one of the most important mechanisms which consume NADPH in *S. cerevisiae* is the reductive amination from 2-oxoglutarate to glutamate catalyzed by NADPH-dependent glutamate dehydrogenases encoded by the *GDH1* and *GDH3* genes (Nissen *et al.*, 2000). In contrast, in *K. lactis* neither null *GDH1* mutants nor null GOGAT mutants show a growth defect phenotype, thus both pathways seem to have a similar role for glutamate biosynthesis (Romero *et al.*, 2000).

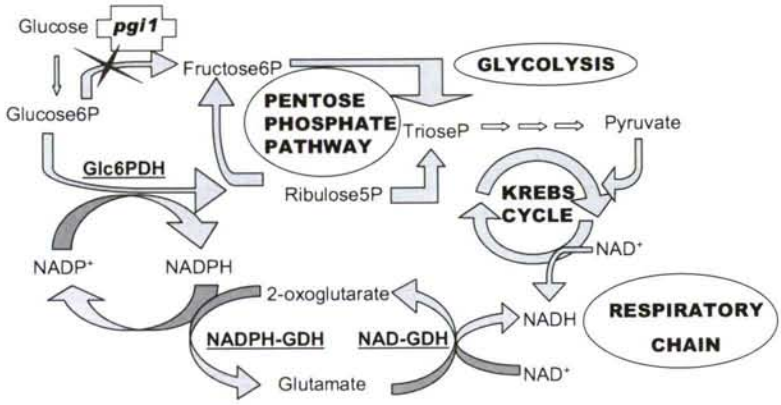

Figure 2: Scheme of the proposed model for *pgi1* suppression by high-level expression of NAD-glutamate dehydrogenase (Modified from Boles *et al.*, 1993)

Overkamp *et al.* (2002) observed in the *K. lactis rag2* mutant a coordinate increase of the mitochondrial NAD(P)-dependent alcohol dehydrogenase gene transcription (*KlADH3*) and a cytosolic NADPH-dependent acetaldehyde reductase activity, both enzymes may constitute an ethanol-acetaldehyde redox shuttle (Fig. 3). It was described that cytosolic alcohol dehydrogenase isoenzymes of *K. lactis* (Adh1p and Adh2p) are strictly NAD-dependent (Bozzi *et al.*, 1997) and the NADPH-dependent acetaldehyde reductase has still not been identified, although ethanol accumulation has been reported in *adh* null *K. lactis* strains (Saliola *et al.* 1994).

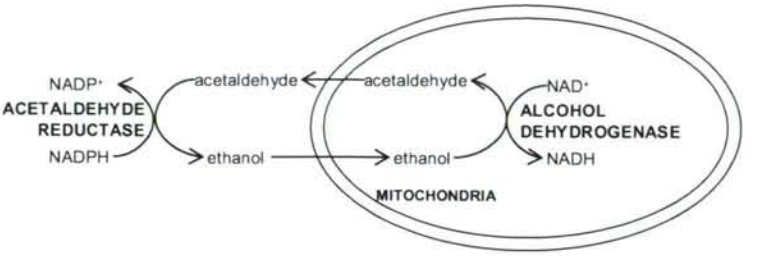

Figure 3: Scheme of the model proposed by Overkamp *et al.* (2002) for NADPH reoxidation through a two-compartment transhydrogenase cycle in the *rag2* mutant.

OXIDATIVE STRESS RESPONSE ENZYMES

The process of respiration requires the uptake of molecular oxygen in which mitochondria consume more than 90% of the total oxygen used by the cell.

While the respiratory chain is necessary for aerobic organisms to produce energy efficiently, it has also meant that they are exposed to the damaging effects of oxygen because oxygen is capable of undergoing excitation or partial reduction to form highly reactive species, including the superoxide anion, hydrogen peroxide or the hydroxyl radical (Turrens, 1997; Møller, 2001). One-electron carriers of the respiratory chain are able to donate one electron to molecular oxygen with the resulting formation of superoxide; the superoxide is dismutated to hydrogen peroxide either catalyzed by superoxide dismutase or spontaneously by the reaction with the protons, and the hydrogen peroxide can be converted to the highly toxic hidroxyl radical in the presence of Fe^{2+} ions by the Fenton reaction. The mitochondria are the major source of ROS in cells (Boveris and Cadenas, 1982; Chance *et al.*, 1979).

The cytocrome bc1 complex has been identified as a site of superoxide generation in mitochondria (Boveris and Cadenas, 1982; Chance *et al.*, 1979). A second site of superoxide production in the mitochondrial electron transport chain is complex I, the rotenone-sensitive NADH:ubiquinone oxidoreductase (Takeshige and Minakami, 1979; Genova *et al.*, 2001) but the complex I is not present in *S. cerevisiae* and *K. lactis* mitochondria. Finally, the external alternative NADH dehydrogenases produce approximately half of the superoxide radicals produced by *S. cerevisiae* mitochondria as a result of transferring electrons to oxygen (Fang and Beattie, 2003). The superoxide radicals cannot permeate the mitochondrial inner membrane, as NADH and NADPH, thus the signals detected in the cytoplasm due to superoxide formation do not represent superoxide released from the mitochondrial matrix. This fact is related to

reports that have provided evidence for a fraction of the Cu,Zn-containing superoxide dismutase in the intermembrane space of yeast (Sturtz *et al.*, 2001).

Oxidative stress in cells is a state in which ROS levels exceed the available antioxidant defences. NADPH is a coenzyme used by important oxidative stress response enzymes as glutathione and thioredoxin reductases; these enzymes oxidize NADPH to $NADP^+$ using it as reducing power source to fight against oxidative stress. Other enzymes are involved in the oxidative stress response such as catalase, superoxide dismutase and thiol-peroxidases.

In the Fig. 4, oxidative stress response pathways are shown in relation with the NADPH production by the pentose phosphate pathway and the ROS production by the mitochondria.

The pentose phosphate pathway is one of the metabolic routes active against oxidative stress in *S. cerevisiae*, supplying NADPH for these enzymes. The activation of glucose 6 phosphate dehydrogenase and other NADPH-dependent response enzymes as glutathione and thioredoxin reductases by oxidative stress was reported by Godon *et al.* (1998). Furthermore Minard and McAlister-Henn (2001, 2005) have demonstrated that mainly the pentose phosphate pathway but also the isocitrate dehydrogenase encoded by *IDP2* gene are necessary for generating the amount of NADPH sufficient enough for the function of thiol-dependent reductases to protect the cell against the oxidative damage. Glutathione reductase, encoded by *GLR1* gene, and thioredoxin reductase, encoded by *TRR1* and *TRR2* genes in *S. cerevisiae* belong to this group of enzymes (Godon *et al.*, 1998). The thiol-dependent mechanisms are completely necessary to protect the cell against reactive oxygen species (ROS) produced in a natural way as subproducts of the metabolic pathways (Minard and McAlister-Henn, 2001).

In *S. cerevisiae* there are two thiol-dependent mechanisms of defence to oxidative stress, glutathione-glutaredoxin and thioredoxin systems, both are represented in the mitochondria by the thioredoxin reductase (Trr2p), one thioredoxin (Trx3p), the glutathione reductase (Glr1p) and one glutarredoxin (Grx5p), and in the cytoplasm by the thioredoxin reductase (Trr1p), two thioredoxins (Trx1p and Trx2p), the glutathione reductase (Glr1p) and four glutarredoxins (Grx1p, Grx2p, Grx3p and Grx4p).

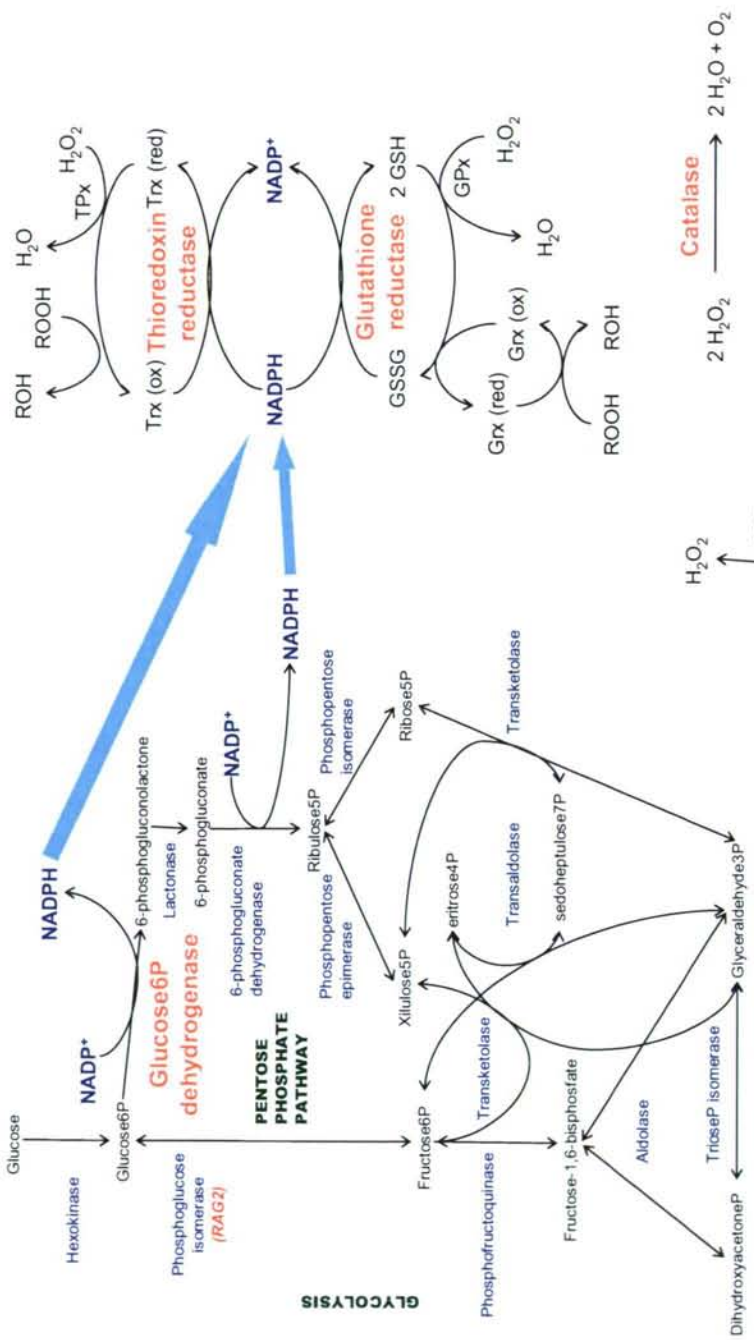

Figure 4: NADPH-dependent oxidative stress response. The enzymes studied in this work are in larger bold font.

Two alternative start translation sites in the *GLR1* gene are the reason that this gene codes for the mitochondrial and cytoplasmic isoforms of glutathione reductase (Outten and Culotta, 2004). The thioredoxin and glutathione-glutaredoxin systems have an overlapping function in redox regulation (Trotter and Grant, 2005). The first evidence came from the identification of the *GLR1* gene in a genetic screening for mutations which confer a requirement for thioredoxins (Muller, 1996). Glr1p and Trr1p are the key regulatory enzymes determining the redox state of the glutathione-glutaredoxin and thioredoxin systems, respectively. The redox states of the mitochondrial and cytoplasmic thioredoxin systems are maintained independently, since loss of one system does not affect the redox state of the other. In contrast, the redox state of the mitochondrial thioredoxin system was found to depend on the presence of an intact glutathione system because the redox state of Trx3p is maintained in the reduced form in mutants lacking components of the cytoplasmic thioredoxin system or *Δtrr2* mutants, but Trx3p is partially oxidized in the *Δtrr2glr1* mutant (Trotter and Grant, 2005). In mammalian cells, Grx2p, which is targeted to either mitochondria or nucleus by an alternative splicing mechanism, can be reduced by both the cytoplasmic and mitochondrial isoforms of thioredoxin reductase (Johansson *et al.*, 2004). However, in *S. cerevisiae* it was described that thioredoxin reductase is unable to reduce glutaredoxins (Holmgren, 1989)

A search in the *K. lactis* whole-genome sequence available in *Génolevures* data base (http://www.cbi.labri.u-bordeaux.fr/Genolevures) for *K. lactis* genes with high similarity to the *S. cerevisiae* oxidative stress response genes shows that some of them do not exist in *K. lactis* and one of them is duplicated in *K. lactis*. Although exceptionally *AHP1* (thiol-specific peroxiredoxin) is duplicated in *K. lactis*, the general tendency is that duplicated genes in *S. cerevisiae* present a single copy in *K. lactis*, as the two genes which code for cytoplasmic-nuclear thioredoxins in *S. cerevisiae* (*TRX1* and *TRX2*) and the genes which code for the cytoplasmic thioredoxin peroxidases (*cTPx1* and *cTPx2*). Moreover, the three *S. cerevisiae* glutathione peroxidases encoded by *GPX1*, *GPX2* and *GPX3* are reduced to two similar proteins in *K. lactis*, and even the *S. cerevisiae* four cytoplasmic-nuclear glutaredoxins encoded by *GRX1*, *GRX2*, *GRX3* and *GRX4* are reduced to two proteins in *K. lactis*. Also this fact occurs with the two genes that code for thioredoxin reductases (*TRR1* and *TRR2*) which respond to different subcellular localization of the proteins in *S. cerevisiae*. The decrease in number of genes of the oxidative stress response in *K. lactis* is another example related to the fact that *S. cerevisiae* is a degenerated tetraploid with many duplicated genes which often seem to be phenotypically redundant (Wolfe and Shields, 1997).

Despite the decreased number of genes coding for oxidative stress response enzymes in *K. lactis* with respect to *S.cerevisiae*, the *K. lactis* genome sequence contains representative genes in every oxidative stress response pathways characterized in *S. cerevisiae* (Huh *et al.*, 2003) and in all cellular compartments where this response takes place, these being: the cytoplasm with genes similar to *GSH1* (γ-L-glutamil-L-cystein synthetase), *GSH2* (glutathione synthetase), *CTT1* (cytoplasmic catalase), *cTPx1* and *GPX3*, the mitochondria with similar genes to *GRX5*, *TRX3*, *SOD2* (mitochondrial superoxide dismutase) and *CCP1* (cytocrome c- peroxidase), the cytoplasm-nucleus with similar genes to *GRX1*, *GRX3*, *TRR1*, *TRX1*, *SOD1* (cytoplasmic superoxide dismutase) and *GPX2*, the peroxisome-mitochondria with a similar gene to *CTA1* (catalase), and the mitochondria-cytoplasm-nucleus with a similar gene to *GLR1*.

In *S. cerevisiae*, the transcription of the *GLR1* and *TRR1* genes increase in response to hydrogen peroxide treatment but the response is higher in *TRR1* than *GLR1*. The transcription of the *TRR2* gene does not increase in response to this treatment (Monje-Casas *et al.*, 2004). It was demonstrated that this response to mild ROS stress is up-regulated by Yap1p (Grant, 2001; Gasch *et al.*, 2000). In this yeast, oxidative stress response has also been related to multistep phosphorelay pathways mediated by the Skn7p transcription factor (Morgan *et al.*, 1997; Lee *et al.*, 1999, Ikner and Shiozaki, 2004).

K. lactis relies predominantly on respiratory metabolism of glucose under aerobic grow conditions, differing with *S. cerevisiae*. In consequence, *K. lactis* has to deal with a higher amount of reactive oxygen species produced by mitochondria than *S. cerevisiae*. Therefore, *K. lactis* constitutes a good model, alternative to *S. cerevisiae*, to study the oxidative stress response mechanisms. However, until this work only one gene directly related to oxidative stress response was characterized in *K. lactis*, *KlYAP1* (Billard *et al.*, 1997), being scarce the knowledge of the oxidative stress response in this yeast.

MITOCHONDRIAL REOXIDATION

The mitochondria is the most important source of ATP in non- photosynthetic eukaryotes, the electrons are lead through the electron transport chain from NADH or NADPH to oxygen generating ATP coupled to proton gradient (Hatefi, 1985)

In contrast to mammals, fungi and plants mitochondria have non-proton-pumping NAD(P)H dehydrogenases for transferring electrons to ubiquinone. These alternative enzymes are single polypeptides localized in the inner membrane facing

either intermembrane space (external dehydrogenases) or mitochondrial matrix (internal dehydrogenases) (Møller and Rasmusson, 1998)

This family of homologous proteins in yeasts shares some similarity with the single-subunit NADH dehydrogenase of *Escherichia coli* (Marres *et al.*, 1991) but these proteins appear to be evolutionarily and structurally distinct from the multisubunit complex I ubiquinone oxidoreductase (Small and McAlister-Henn, 1998)

During respiratory growth, the surplus of NADH can be reoxidized in the mitochondrial respiratory chain obtaining the ATP necessary for growth. *S. cerevisiae* utilizes an internal dehydrogenase (Ndi1p) and two external dehydrogenases (Nde1p and Nde2p) (Marres *et al.*, 1991; Luttik *et al.*, 1998; Small and McAlister-Henn, 1998)

It has been proposed that the presence of external NADH dehydrogenases with the capacity for direct oxidation of cytoplasmic NADH has a close connection with the malate-aspartate shuttle cycle not being functional in yeast (De Vries and Marres, 1987; Hollenberg *et al.*, 1970) However, in *S. cerevisiae* other shuttles have been reported to be functional such as glycerol 3-phosphate dehydrogenase (Larsson *et al.*, 1998), ethanol-acetaldehyde (Nissen *et al.*, 1997, Bakker *et al.*, 2000) and malate-oxalacetate (Palmieri *et al.*, 1999)

Ndi1p is not essential for the respiratory growth in *S. cerevisiae* because of the ethanol-acetaldehyde redox cycle activity, which transfer redox equivalents from mitochondria to cytoplasm (Marres *et al.*, 1991). *S. cerevisiae* has two genes encoding for NADH external dehydrogenase enzymes, *NDE1* and *NDE2* (Luttik *et al.*, 1998; Small and McAlister-Henn, 1998), both genes were identified in base to their homology with the *NDI1* gene and the alignment of the three protein sequences revealed that both Nde1p and Nde2p have a N-terminal extension which is absent in Ndip (Small and McAlister-Henn, 1998).

During glucose growth, the transcription levels of the *NDE1* and *NDE2* genes are increased when glucose decreases and the cells start to use the ethanol previously produced. In this moment, *NDE2* gene is induced much stronger, seven times, than *NDE1* gene, two times (De Risi *et al.*, 1997). Both are genes typically aerobic, whose mRNA levels are increased when the cells are changed from anaerobic to aerobic conditions. In this case the *NDE1* transcription levels are increased four times and the *NDE2* transcription levels fourteen times (Ter Linde *et al.*, 1999). However, Nde1p is physiologically more important than Nde2p (Bakker *et al.*, 2000)

Grandier-Vazeille *et al.* (2001) have identified in *S. cerevisiae* a supramolecular structure with NADH dehydrogenase activity. It is a mitochondrial complex (Fig. 5) with five enzymes whose active centres are orientated to intermembrane space. There are two NADH external dehydrogenases (Nde1p and Nde2p), glycerol 3-phosphate

dehydrogenase (Gut2p) and D- and L-lactate dehydrogenases (Dld1p and Cyb2p). Moreover this supramolecular structure has other enzymes with the active centres directed to the mitochondrial matrix. There is one NADH dehydrogenase (Ndi1p), two putative flavoproteins (YOR356Wp and YPR004Cp), the acetaldehyde dehydrogenase (Ald4p) and four enzymes of the Krebs cycle: malate dehydrogenase (Mdh1p), citrate synthase (Cit1p), succinate dehydrogenase (Sdh1p) and fumarate hydratase (Fum1p). Also, the cytocrome bc_1 and cytocrome c oxidase complexes are associated as a larger single supracomplex in the *S. cerevisiae* mitochondria (Cruciat *et al.*, 2000).

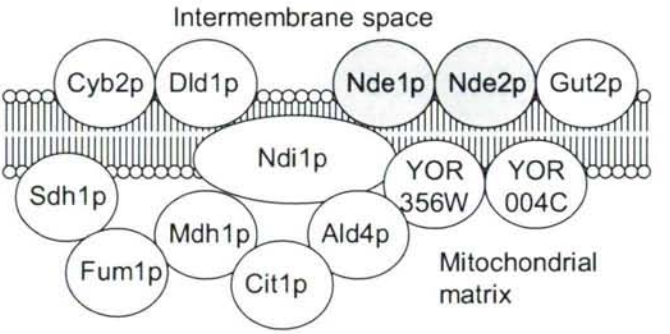

Figure 5: Scheme of the components of the mitochondrial dehydrogenase complex (Modified from Grandier-Vazeille *et al.*, 2001)

In *Yarrowia lipolytica*, a strictly aerobic yeast, the *NDH2* gene which codes for an external non-proton-pumping alternative dehydrogenase was characterized. The Ndh2p is NADH specific, identified as being the single alternative dehydrogenase of *Y. lipolytica* because mitochondria from a *ylndh2* mutant were fully sensitive to piericidin A, an inhibitor of proton translocating complex I (Kerscher *et al.*, 1999). These authors also demonstrated that the complex I is the single internal NADH:ubiquinone oxidoreductase in *Y. lipolytica* mitochondria. However, in *Y. lipolytica* whole-genome there is another sequence similar to the *NDE1* gene from *Neurospora crassa*, which, in function of these results, seems not to be functional.

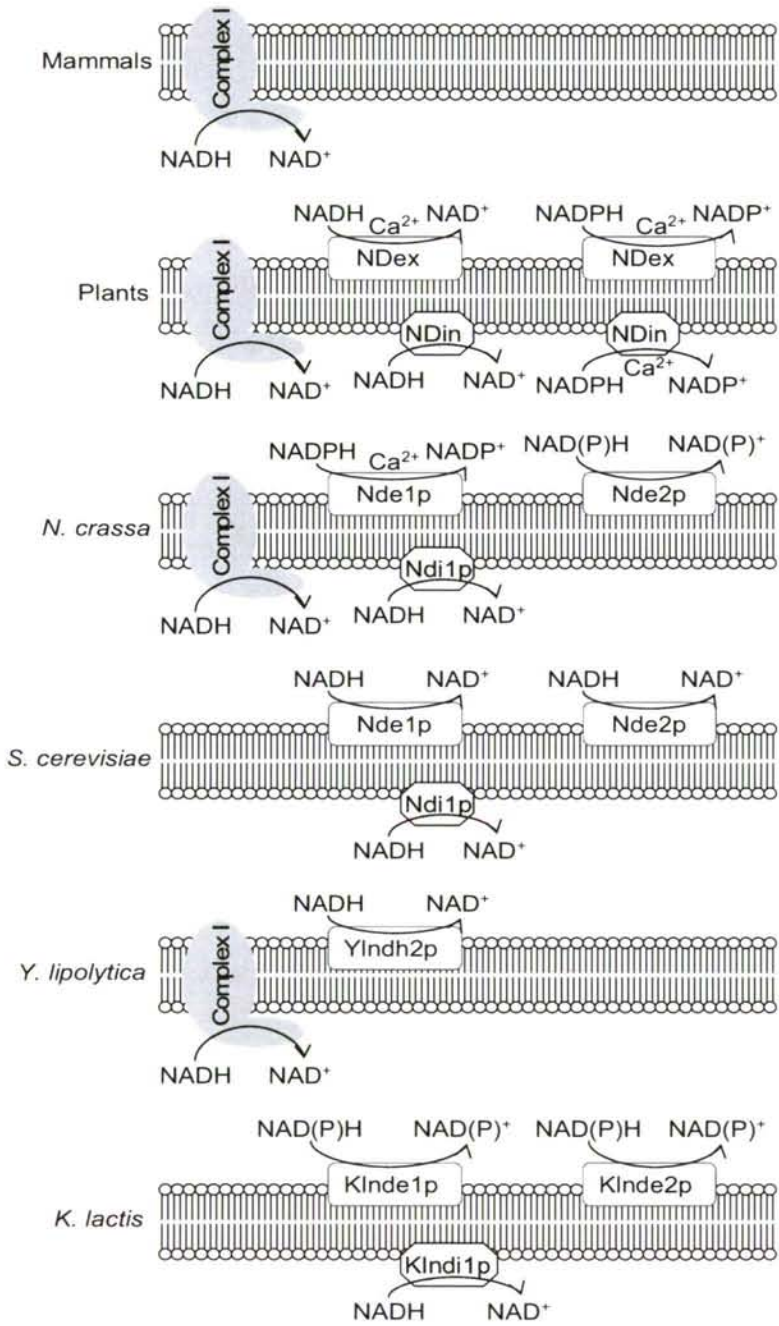

Figure 6: Scheme of the NAD(P)H dehydrogenases present in the respiratory chain of mitochodria from mammals, plants, *N. crassa*, *S. cerevisiae*, *Y. lipolytica* and *K. lactis*. Above intermembrane space and bellow mitochondrial matrix. Modified from (Møller, 2001) adding *Y. lipolytica* (Kerscher, 2000) and *K. lactis* (this work)

The NADPH external dehydrogenase activity has been demonstrated in plants, where it is common (Möller and Lin, 1986), filamentous fungi (Melo *et al.*, 2001) and in yeasts, specifically in the mitochondria from *Candida utilis* (Bruinenberg *et al.*, 1985b; Van Urk *et al.*, 1989) where it was established that the NADPH is oxidized in the respiratory chain, but only in the phosphorilation II and III sites (De Vries and Marres, 1987), from *Saccharomyces carlsbergensis* (Schuurmans Stekhoven, 1966) and from *K. lactis* (Overkamp *et al.*, 2002). But hitherto only five genes have been identified which encode for NADPH external mitochondrial dehydrogenases, two of them in *Neurospora crassa* (Melo *et al.*, 2001; Carneiro *et al.*, 2003), one from *Solanum tuberosum* (Michalecka *et al.*, 2004) and the others from *K. lactis* (this work). In Fig. 6 a scheme is shown about the external alternative dehydrogenases in different organisms.

Although the single plant gene hitherto characterized is *StNDB1* which codes for an external calcium-dependent NADPH-specific alternative dehydrogenase (Michalecka *et al.*, 2004), it is known that all plant mitochondria investigated are able to oxidize external NADH and NADPH. These substrates are oxidized by two distinct calcium-dependent enzymes because mitochondrial NADH oxidation can be induced without inducing NADPH oxidation, the relative activities of NADH and NADPH oxidation vary in mitochondria from different tissues or cell cultures and NADPH oxidation is much more sensitive to inhibition by diphenyleneiodonium (DPI) than is NADH oxidation (Roberts *et al.*, 1995). Also, plant mitochondria have internal alternative dehydrogenases, some are NADH-specific and other NADPH-specific, and only the latter are dependent on calcium for activity (Møller, 2001).

In the filamentous fungus *N. crassa*, three genes which code for mitochondrial alternative dehydrogenases have been characterized, two of them, *NDE1* and *NDE2*, code for external dehydrogenases (Melo *et al.*, 2001; Carneiro *et al.*, 2003) and the other, *NDI1*, codes for an internal dehydrogenase (Duarte *et al.*, 2003). The Ndi1p, which is not calcium-dependent, oxidizes NADH specifically (Duarte *et al.*, 2003) whereas the external dehydrogenases can oxidize NADPH (Melo *et al.*, 2001; Carneiro *et al.*, 2003). The Nde1p oxidizes low levels of NADPH at basic pH and is dependent of calcium (Melo *et al.*, 2001) and the Nde2p is the main external alternative dehydrogenase which is capable to oxidize both NADH and NADPH and is not calcium-dependent (Carneiro *et al.*, 2003).

Unlike *N. crassa*, but as with *S. cerevisiae*, the absence of complex I from *K. lactis* mitochondria was evidenced long ago (Josep-Horne *et al.*, 2001). However, the external alternative dehydrogenases were completely unknown up to this work.

OBJECTIVES

The aim of this thesis is to study the molecular mechanisms of cytosolic NADPH reoxidation in *Kluyveromyces lactis*, specifically:

1. The global response in *K. lactis* transcriptome to an increase in cytosolic NADPH production due to the obligated glucose utilization through the pentose phosphate pathway.
2. The operation of a transhydrogenase cycle mediated by glutamate dehydrogenase isoenzymes in cytosolic NADPH reoxidation in *K. lactis*.
3. The NADPH-dependent oxidative stress response in *K. lactis*.
   - 3.1. Cloning, characterization and functional analysis of the *KlGLR1* and *KlTRR1* genes which code for glutathione and thioredoxin reductases.
   - 3.2. The implication of glutathione and thioredoxin reductase activities in cytosolic NADPH reoxidation.
   - 3.3. The involvement of the enzymes glutathione reductase, thioredoxin reductase, catalase and glucose 6 phosphate dehydrogenase in the responses to oxidative stress and to shift aerobiosis/hypoxia.
   - 3.4. Subcellular localization of glutathione and thioredoxin reductases.
4. The external alternative dehydrogenases from *K. lactis* mitochondria and their role in the cytosolic NADPH reoxidation.
   - 4.1. Cloning and characterization of the two genes (*KlNDE1* and *KlNDE2*) which code for mitochondrial external alternative dehydrogenases.
   - 4.2. Heterologous expression in *S. cerevisiae* of *KlNDE1* and *KlNDE2* genes.
   - 4.3. Construction of the null mutant in *KlNDE1* and assessment of the associated phenotype.
   - 4.4. Construction of the double null mutant in *RAG2* (phosphoglucose isomerase) and *KlNDE1* genes and evaluation of the associated phenotype.
   - 4.5. The activity and regulation of the two external alternative dehydrogenases in *K. lactis* mitochondria.

OUTLINE OF THIS THESIS

The relative flux of glucose through the glycolysis or the pentose phosphate pathway with the consequent NADPH production is one of the fundamental mechanisms which regulate the preferential use of respiration or fermentation in yeasts. As the efficacy in the NADPH reoxidation is a key point for the capacity of utilization of the pentose phosphate pathway, this thesis deals with the study of the cellular mechanisms involved in cytosolic NADPH reoxidation in the respiratory yeast *Kluyveromyces lactis*.

Chapter 1 describes a new method for isolating clones from a library when only partial sequences of the genes of interest are available. This method, called Discriminating Clusters by Polymerase Chain Reaction (DCbyPCR), starts amplifying a library which contains our gene of interest. The library is spread plated to obtain individual bacterial colonies. After that, the presence of the clone of interest is determined in plasmid DNA extracted from pools of these bacterial colonies using PCR, the pool giving a positive band by PCR is selected and then its components are used separately as templates for new PCR reactions until arriving at the isolated clone of interest. In this thesis three clones were obtained by this method, the plasmids carrying *KlGLR1* (glutathione reductase), *KlTRR1* (thioredoxin reductase) and *KlNDE1* (mitochondrial external alternative dehydrogenase 1).

Chapter 2 is about the isolation, characterization and functional analysis of two clones from a *K. lactis* library, one of them carries the *KlGLR1* gene which codes for glutathione reductase and the other carries the *KlTRR1* gene which codes for thioredoxin reductase. Differences in the oxidative stress response are shown in *K. lactis* with respect to the more widely studied *S. cerevisiae*. The transcription levels of *KlTRR1* increase in response to hydrogen peroxide treatment whereas *KlGLR1* transcription levels do not. This fact is supported by the corresponding enzyme activity measurements in *K. lactis*. The presence of a functional consensus for Yap1p binding in the *KlTRR1* promoter, being absent in the *KlGLR1* promoter, may explain this different regulation.

Chapter 3 shows a genome-wide analysis of *K. lactis* in wild type and *rag2* mutant strains using heterologous DNA-arrays from *S. cerevisiae*. The goal was to look for pathways induced in the *rag2* mutant growing in glucose that could be involved in NADPH reoxidation. In the *rag2* (phosphoglucose isomerase) mutant all the glucose flux is through the pentose phosphate pathway thus producing a cytosolic NADPH surplus. From the results we outline four main observations. First, the phosphoglucose isomerase mutation causes very little disturbance upon transcription of genes related to the pentose phosphate pathway when glucose is the carbon source in *K. lactis* This result was corroborated by enzyme assays of glucose 6-phosphate dehydrogenase

showing that this activity is similar in the wild type and *rag2* strains of *K. lactis* and also that it is significantly higher in *K. lactis* than in *S. cerevisiae*, which agrees with previous reports about higher glucose 6-phosphate dehydrogenase activity in Crabtree-negative versus Crabtree-positive yeasts. Second, clustering analysis of gene expression applied to functional groups reveals that none biosynthetic pathway using NADPH is clearly increased in the transcriptome of the *rag2* mutant growing in glucose compared to fructose and that the metabolism of the *rag2* mutant growing in fructose is less respiratory and energy generating than in glucose, these data are supported by the fact that the growth rate of the *rag2* mutant in glucose is higher than in fructose. Third, the individual analysis of the group of genes which code for NAD- and NADPH-dependent glutamate dehydrogenases, corroborated by northern blotting and enzyme assays, discards the operation of these isoenzymes as a transhydrogenase cycle in the *K. lactis rag2* mutant growing in glucose. And fourth, a moderate increase in transcript levels of some genes involved in the defence against oxidative stress is observed when comparing the *rag2* mutant in glucose compared to fructose cultures. This fact may be related to the high activity of the respiratory chain when the *rag2* mutant is grown in glucose, which could increase intracellular levels of reactive oxygen species that could set up the oxidative stress response.

Chapter 4 describes the isolation and characterization of the *KlNDE1* gene from *K. lactis*, which codes for a mitochondrial alternative dehydrogenase facing the intermembrane space. The transcriptional regulation of this gene and the substrate specificity of the protein compared to the *S. cerevisiae* counterparts provide distinctive keys between *S. cerevisiae* (fermentative) and *K. lactis* (respiratory). First, there are no differences in *KlNDE1* transcription levels when *K. lactis* is cultured in glucose (fermentable carbon source) or lactate (non-fermentable carbon source), whereas the genes which code for mitochondrial external alternative dehydrogenases in *S. cerevisiae* are repressed by glucose. Second, Klnde1p uses NADPH as substrate whereas the external alternative dehydrogenases of *S. cerevisiae* are NADH-specific. The ability of Klnde1p to reoxidize the NADPH produced in the pentose phosphate pathway is inferred because *KlNDE1* overexpression restores the growth in glucose media of the *S. cerevisiae pgi1* mutant which is obligated to re-route all glucose through the pentose phosphate pathway. And third, the involvement of this protein in the cytosolic NADPH reoxidation in *K. lactis* cells, is deduced because the *K. lactis rag2* mutant has higher transcription levels of *KlNDE1* when it is cultured in glucose than the wild type strain also cultured in glucose or when this mutant is cultured in fructose. Thus, the capacity to oxidize NADPH by mitochondrial external alternative dehydrogenases allows a higher use of the pentose phosphate pathway in yeasts.

Chapter 5 is a mini-review about the research done in our laboratory and others looking for the nature of the molecular mechanisms involved in the reoxidation of the cytosolic NADPH produced as a consequence of the operation of the pentose phosphate pathway in *K. lactis*. These mechanisms were envisaged as a key feature leading to the metabolic differences between *S. cerevisiae* and *K. lactis*. In this chapter it is proposed that the mitochondrial external dehydrogenases of *K. lactis* represent the main pathway for the reoxidation of the cytosolic NADPH, supporting the high activity of the pentose phosphate pathway and, therefore, allowing the growth in glucose of the *rag2* mutant. Also, the activity of the glutathione reductase is proposed to contribute to the adaptation of the *rag2* mutant to glucose catabolism based exclusively on the pentose phosphate pathway.

Chapter 6 deals with the interplay between oxidative stress response and cytosolic NADPH turnover in *K. lactis*. It is shown that there is a relationship between the prevalence of respiratory metabolism and the resistance to oxidative stress in yeasts, which is attributed to the fact that the mitochondrial respiratory chain is the major site of ROS production. Thus, with glucose as carbon source, the respiratory *K. lactis* is more resistant to oxidative stress than the fermentative *S. cerevisiae* and similarly, the *K. lactis rag2* mutant, characterized by an increased activity of the respiratory chain, is more resistant to oxidative stress than the wild type strain. We have demonstrated that this higher resistance of the *rag2* mutant is supported by increased levels of activity of glutathione reductase and catalase. The glutathione reductase also contributes to regenerate $NADP^+$ for the pentose phosphate pathway (glutathione reductase and glucose 6 phosphate activity are correlated) but this enzyme activity, even if overexpressed, is not enough to allow the growth on glucose of the *rag2* mutant when the mitochondrial reoxidation of cytosolic NADPH is blocked by antimycin A or by oxygen deprivation. Regarding this, it has been reported that the external mitochondrial NADPH dehydrogenase from plants has the greater capacity for NADPH oxidation when compared to the pool of cytosolic NADPH-utilizing enzymes. This chapter also demonstrated that thioredoxin reductase and catalase are involved in the oxidative stress response against hydroperoxides, but neither glutathione reductase nor glucose 6-phosphate dehydrogenase are, the activity of these two enzymes being dependent on culture dissolved oxygen levels. We propose that in yeasts the diverse patterns of respiro-fermentative metabolism are related to differences in the oxidative stress response through variations in the activity of the pentose phosphate pathway and the mechanisms used for NADPH reoxidation.

Chapter 7 describes the characterization of the second external alternative dehydrogenase from *K. lactis* mitochondria (Klnde2p) and the evaluation of its relative

importance in cytosolic NADPH reoxidation compared to Klnde1p. For the latter purpose, we built two null mutants: *Δklnde1* and *Δklnde1rag2*. The second mutant is defective in the growth in glucose, therefore Klnde2p as single external dehydrogenase is unable to reoxidize efficiently the NADPH generated by the pentose phosphate pathway when this route become essential for the growth in glucose because glycolysis is blocked. In contrast the single mutant, *Δklnde1*, does not present any deficiency in growth in different carbon sources or in NAD(P)H oxidation by isolated mitochondria. In this regard, we studied the role of four factors in the modulation of the activity of the two external alternative dehydrogrenases from *K. lactis* mitochondria: the pH, the down-regulation by glucose, the calcium effect and the cytosolic NADPH concentration. First, we measured the two enzymes activities at different pH values and we concluded that both enzymes show optimal activity at the same range of pH. As far as down-regulation by glucose is concerned, we measured NADH and NADPH oxidation rates by isolated mitochondria from cells, wild type and *Δklnde1* mutant, cultured in lactate and glucose media and the results demonstrated that down-regulation by glucose is not operating on *KlNDE2* gene, because the oxidation rates are even higher in glucose than in lactate. With regard to calcium effect, the calcium does not regulate the activity of Klnde2p in isolated mitochondria although this protein has a calcium binding motif weakly predicted by the MotifScan program. Finally, we propose that the cytosolic NADPH concentration modulates the relative importance of these two external alternative dehydrogenases in the reoxidation of the NADPH from the pentose phosphate pathway, because the NADPH affinity is higher in Klnde1p than in Klnde2p. We demonstrated that the K_M (NADPH) of Klnde2p is two-fold higher than the K_M (NADPH) of Klnde1p. Moreover, the heterologous expression in the *S. cerevisiae pgi1* mutant supports this conclusion because the overexpression of Klnde1p restores the *pgi1* growth in media with glucose concentration until 2% whereas the overexpression of Klnde2p only restores the *pgi1* growth in media with glucose concentration until 0.4%.

Chapter 8 is about a new subject of investigation which is emerging in our laboratory. We describe preliminary studies about the subcellular localization of glutathione and thioredoxin reductases from *K. lactis* by means of the overexpression in *S. cerevisiae* of the genes which code for these reductases fused to the green fluorescent protein. Our aim is, initially, to elucidate whether a single gene can give rise to both the cytoplasmic and mitochondrial isoforms of the proteins.

CHAPTER 1

Cloning genes from a library using a clustering strategy and PCR

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Molecular Biotechnology (2004) 26: 35-38

SUMMARY

A new polymerase chain reaction (PCR)-based method is described for the isolation of clones of interest from a library when only part of a sequence is available. In actuality, this occurs with many genomes that have been partially sequenced using a random strategy. The method presented here, discriminating clusters by PCR (DCbyPCR), is a non-radioactive and improved alternative to colony hybridization.

INTRODUCTION

Since the implementation of recombinant deoxyribonucleic acid (rDNA) technology, cloning the gene of interest constitutes the crucial first step in its use for basic or applied purposes. Unless the gene could be cloned by complementation of a mutant, it is essential to have some sequence information available, however limited it may be. Nowadays, although few genomes have been completely sequenced, for most a partial random sequencing strategy is used (Feldman, 2000); databases contain plenty of random sequenced tags (RSTs). Usually the only resources available to clone genes are these RSTs of the gene of interest or of homologous genes of related species. A classical approach in these cases is colony hybridization with labeled probes of the known sequence or of conserved regions. However, this technique is tedious and often gives rise to many false-positive candidates.

We propose a method, discriminating clusters by PCR (DCbyPCR), which from a library allows the isolation of clones containing the gene of interest in less than 1 week. A brief description is presented and includes as an example the cloning of a gene of the yeast *Kluyveromyces lactis* (*KlGLR1*) from a genomic library constructed in the shuttle plasmid KEp6 (Wésolowski-Louvel *et al.*, 1988). The procedure may be adapted to other organisms and types of libraries. Basically, using polymerase chain reaction (PCR), the presence of the clone of interest is determined in clusters of plasmid deoxyribonucleic acid (DNA) extracted from pools of the bacterial colonies bearing the clones of the library. In each step, the cluster giving a positive band by PCR is selected, and then the components of this cluster are used separately as templates for new PCR reactions until arriving at the isolated clone of interest. An important advantage of the method here proposed is that it does not require re-amplification of the library during the screening, that is, the library is plated out once and then analysis of replica plates is used rather than several rounds of plasmid isolation, PCR-analysis, serial dilution, and retransformation. The effectiveness of the DCbyPCR method was demonstrated in our research group by isolating nine clones

containing genes of interest from two different libraries using the protocol here described. Previous attempts to clone several among these genes by other procedures, including colony hybridization, failed. As an alternative to colony hybridization, DCbyPCR is -in our hands- a more successful method, being non-radioactive, faster and easier. A scheme is shown in Fig. 1.

MATERIALS

1. *Escherichia coli* DH-10B was used for plasmid amplification.
2. LBA (1% bacto-tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose, ampicillin 40 mg/L) was used as culture medium for the bacteria.
3. Electroporation solution: 10% glycerol.
4. Miniprep solutions: 50 mM glucose, 10mM ethylenediaminetetraacetic (EDTA), 25mM Tris-HCl, pH 8.0; 0.2M NaOH, 1% sodium dodecyl sulphate (SDS); potassium acetate, pH 4.8, added to 60 mL of 5M potassium acetate, 11.5 mL of glacial acetic acid and 28.5 mL of dH_2O.
5. Ribonuclease solution: 0.6% bovine pancreatic ribonuclease A (Sigma) in 10 mM Tris-HCl pH = 8.5, treated at 100ºC for 18 minutes to remove desoxyribonuclease activity. 1 μL was added to 1 mL of DNA miniprep.
6. PCR-mix: 2.5 μL 5x buffer (Roche), 0.7 μL 2.5mM dNTPs, 1μL DNA template, 30 pmol each primer, 1 U *Taq* polymerase (Roche), dH_2O until 25 μL of reaction.
7. Electrophoresis gels: 1% agarose in TAE buffer with 0.5 μg ethidium bromide/mL
8. TAE buffer: 0.04M Tris-acetate, 0.1mM EDTA and 30 mM acetic acid.
9. Loading buffer: 0.25% bromophenol blue and 0.25% xylene cyanol in 50% glycerol.

METHODS

1. It is necessary to start with a library containing the gene of interest and a pair of primers designed to amplify by PCR a part of the sequence of this gene. In our example, a *K. lactis* partial sequence of a gene homologous to the *Saccharomyces cerevisiae GLR1* was used to design a pair of primers that amplify a band of 789 bp. The sequence was available in the nucleotide databases (Accession AL429479, AL429280, AL427879) submitted by Genoscope (www.genoscope.cns.fr) as part of a random genomic sequencing program.

   Amplification must be specific. The first precaution is to verify if the primers anneal in the vector used to construct the library, thus giving unspecific bands by PCR (Fig. 1, *no. 1*). A PCR with genomic DNA as template may be used as a control. In addition, the band of the expected size obtained in the PCR with the library as template may be sequenced.

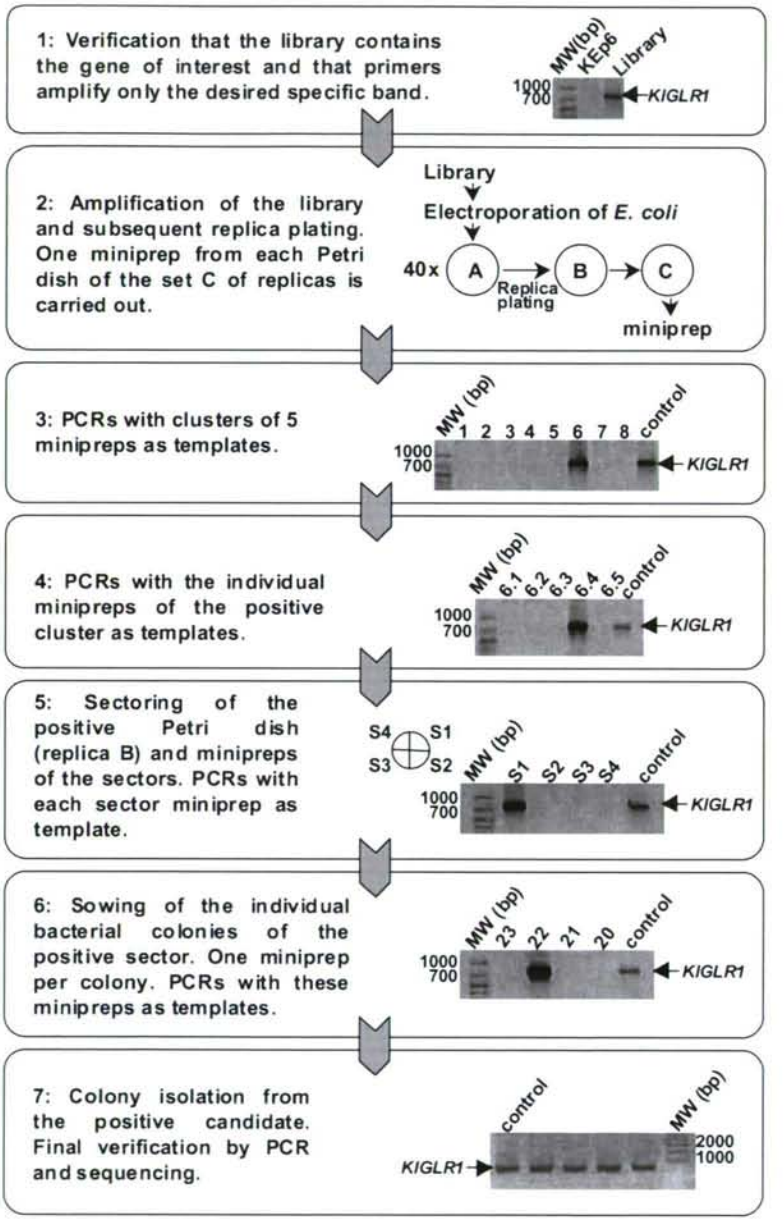

Figure 1: Scheme of the discriminating clusters by PCR (DCbyPCR) method, showing the cloning of the *KIGLR1* gene as an example. The PCR products were visualized on ethidium bromide-stained agarose gels. Negative images of gel photographs are shown.

2. Day 1: The library is spread plated to obtain individual bacterial colonies. The number of colonies depends on the size of the genome and of the inserts of the library. In our example, we obtained about 40000 individual bacterial colonies by electroporation, distributed in 40 Petri dishes. Day 2: Individual Petri dishes containing the bacterial colonies are replicated twice with velvets (Miller, 1992). Day 3: Cells on each Petri dish of the second set of replicas (C) are eluted with approximately 2ml of LBA by repeated washing over the agar surface until all of the colonies are dispersed into the liquid. The eluates are directly used, without incubation, to extract plasmid DNA by the alkaline lysis method (Sambrook *et al.*, 1989). There is one miniprep per Petri dish (Fig. 1, *no. 2*).
3. Aliquots from five minipreps are mixed and the eight clusters of five thus obtained are used as templates for PCR reactions with the primers that specifically amplify a part of the gene of interest. In our example, a positive band of the expected size was obtained in cluster 6 (Fig. 1, *no. 3*).
4. New PCR reactions are performed with the same primers but using as templates the five individual minipreps that were previously mixed in the cluster that gave the positive band. The miniprep that gives a band of the expected size allows the identification of the Petri dish containing the clone of interest. In our example, it was Petri dish 6.4 (Fig. 1, *no. 4*).
5. Day 4: This Petri dish is analyzed by sectoring, that is, it is divided into several parts and plasmid DNA is extracted from all of the bacterial clones in each part as described in step 2. The first replica (*no. 2*) is used for this. There is one miniprep per sector of the Petri dish. PCR reactions are carried out using each miniprep as template. The miniprep that gives a band of the expected size allows the identification of the part of the Petri dish containing the clone of interest. In our example, we divided the replica *no. 2* of Petri dish 6.4 into four sectors; a positive band was obtained in sector 1 (Fig. 1, *no. 5*).
6. The individual bacterial colonies of this part of the original Petri dish (6.4 A) are picked and used to inoculate cultures for minipreps.

   Day 5: PCR reactions are performed using these individual minipreps as templates. The miniprep that gives a band of the expected size allows the identification of the bacterial colony containing the clone of interest. If many colonies are present in the positive sector of the Petri dish, the strategy of clustering the minipreps for the PCRs may be used also in this step. In our example, about 200 colonies were present in the positive sector. A positive band was obtained with the miniprep of colony 22 (Fig. 1, *no. 6*).

7. Day 6-7: The final step is to isolate colonies (Miller, 1992) from the candidate selected and to check them using PCR and by sequencing the plasmid DNA extracted. In our example, all the clones isolated from colony 22 that were analyzed gave the positive band by PCR (Fig. 1, *no. 5*). Sequencing one of these clones confirmed that it contained the full-length gene we were searching for: *KIGLR1*. The sequence was submitted to the EMBL/GenBank/DDBJ databases (accession number AJ504414).

Acknowledgments

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CHAPTER 2

Isolation and characterization of two nuclear genes encoding glutathione and thioredoxin reductases from the yeast *Kluyveromyces lactis*

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SUMMARY

Response to oxidative stress has been hitherto scarcely studied in the respiratory yeast *Kluyveromyces lactis*. The genes coding for reductases of glutathione and thioredoxin, *KlGLR1* and *KlTRR1*, respectively, have been cloned and characterized in this work. H_2O_2 treatment increased transcription and enzyme activity of *KlTRR1* but not of *KlGLR1*, suggesting a different situation from that reported for the fermentative yeast *Saccharomyces cerevisiae*. A consensus for Yap1p binding is functional in the *KlTRR1* promoter.

INTRODUCTION

Oxygen-utilizing cells have evolved defense mechanisms to protect against the toxicity of the highly reactive oxygen species (ROS) that are formed as a consequence of aerobic metabolism but also by exposure to hyperoxia, radiation, light, metals and redox-active drugs. Oxidative stress in cells is a state in which ROS levels exceed the available antioxidant defenses.

An eukaryote model used for studying cellular responses to oxidative stress is the yeast *Saccharomyces cerevisiae* (Jamieson, 1998; Koerkamp *et al.*, 2002). Glutathione reductase (Glr1p) and thioredoxin reductase (Trr1p) of *S. cerevisiae* are members of the termed H_2O_2 stimulon, a group of proteins that exhibit 2-15-fold increases in cellular levels following exposure to exogenous H_2O_2 (Godon *et al.*, 1998). Reduced glutathione and thioredoxin are used as cofactors by a number of enzymes in various detoxification reactions and are important agents involved in keeping a reduced state (Koerkamp *et al.*, 2002; Grant, 2001). Particularly, thioredoxin has been proposed to play a key role in controlling the oxidative state of crucial cysteine residues in the transcription factor Yap1p, which mediates induction of genes involved in the oxidative stress response (Kuge *et al.*, 2001).

Kluyveromyces lactis is an important industrial yeast that is related to *S. cerevisiae* but relies predominantly on respiratory, rather than on fermentative, metabolism of glucose under aerobic growth conditions (González Siso *et al.*, 2000). This respiratory yeast is an alternative model to study oxidative stress since increased ROS formation during respiratory *versus* fermentative growth has been suggested (Cabiscol *et al.*, 2000). However, until this work, the only *K. lactis* gene involved in the oxidative stress response that had been characterized is *YAP1* (Billard *et al.*, 1997). We hereby report the isolation and characterization of the *K. lactis* genes, *KlGLR1* and *KlTRR1*, which code for reductases of glutathione and thioredoxin, respectively.

Expression of these genes under H_2O_2-induced stress reveals differences with the *S. cerevisiae* counterparts.

RESULTS AND DISCUSSION

A *K. lactis* genomic library in the shuttle plasmid KEp6 (Wésolowski-Louvel *et al.*, 1998) was used to search for clones containing genes homologous to the *S. cerevisiae GLR1* and *TRR1* genes by the PCR-based strategy described in (Díaz Prado *et al.*, 2004). About 40,000 bacterial colonies were screened using oligonucleotides that amplified small partial sequences available in GenBank databases (AL429479 and AL425573, respectively).

Two clones were isolated and sequenced by primer walking on both strands using an automated system. The data confirmed the homology of these genes, termed *KlGLR1* and *KlTRR1*, to those previously cloned from other organisms. The sequences have been submitted to EMBL/GenBank/DDBJ databases (accession numbers AJ504414 and AJ504413, respectively). The putative protein Klglr1p shows 73% identity with the *S. cerevisiae* homologue Glr1p. The putative protein Kltrr1p shows 80% identity with the *S. cerevisiae* homologue Trr1p and 76% with the isoform Trr2p.

To test the functional significance of *KlGLR1* and *KlTRR1*, they were overexpressed in the *K. lactis* strain MW190-9B (*MATa, lac4-8, uraA, Rag+*). The lithium acetate procedure was used for transformation with the plasmids (Gietz and Woods, 1994). For the determination of enzyme activities, protein extracts were prepared as follows: Cultures were grown in Erlenmeyer flasks at 30°C and 250 rpm in synthetic complete medium (CM) without uracil up to OD_{600}=0.6. The cells from 400 mL of culture were collected by centrifugation and resuspended in 1 mL of buffer A (0.2 M Tris-HCl pH=7.8, 0.3 M $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol) per gram of wet weight. Cells were broken by vortexing with glass beads in 20-second pulses. The supernatant of a centrifugation at 8000 rpm for 15 minutes was immediately used. All steps were performed in cold. Protein concentration was measured by the method of Bradford (1976) using bovine seroalbumin as a standard. Glutathione reductase and thioredoxin reductase activities were determined as described in Smith *et al.* (1988) and Holmgren and Björnstedt (1995), respectively. Enzyme units (E.U.) are defined as μmol TNB (5´-thionitrobenzoic acid)/ minute × mg protein, under assay conditions. Protein extracts were prepared from four independent transformants. Up to four enzyme activity measurements were taken for each protein extract and the results averaged. Throughout the paper, the statistical significance of differences found between means was evaluated at the 95% confidence level by the ANOVA and

Multiple Comparisons Tests using StatGraphics Plus software. The crude extract of the strain transformed with the *KlGLR1* gene showed an 8-fold increase in glutathione reductase activity (3.77 ± 0.83 E.U., mean ± standard deviation, *n*=16) when compared to wild type (untransformed MW190-9B strain) levels (0.47 ± 0.04 E.U., mean ± standard deviation, *n*=4). These results confirm that *KlGLR1* codes a glutathione reductase. However, the crude extract of the strain transformed with the *KlTRR1* gene did not show an increase in thioredoxin reductase activity (0.33 ± 0.08 E.U., mean ± standard deviation, *n*=10) when compared to wild type levels (0.41 ± 0.09 E.U., mean ± standard deviation, *n*=3). The characteristics of transcriptional regulation of the *KlTRR1* gene by H_2O_2 (described below) explain this result.

To further study the physiological significance of Kltrr1p, the protein was expressed in bacteria and purified. The pET system supplied by Novagen, specifically pET-21d vector and BL21 (DE3) bacterial cells, was used to express the Kltrr1p without the 29 N-terminal residues and with a C-terminal six-histidine tag. After IPTG induction, transformed cells were harvested, resuspended in lysis buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 0.1% Triton X-100) and disrupted by sonication. After centrifugation, the soluble fraction was collected, and the histidine-tagged protein was purified using Ni-affinity chromatography (Ni-NTA agarose). The purified protein was eluted in 50 mM NaH_2PO_4 pH 7.5, 300 mM NaCl and 100 mM imidazole. From SDS-PAGE (data not shown), we estimated that the protein was ~90% pure and had a molecular mass in good agreement with the molecular mass predicted from the sequence. The purified protein showed a thioredoxin reductase-specific activity of 8.38 ± 2 E.U. (mean ± standard deviation, *n*=4). These results confirm that *KlTRR1* codes a functional thioredoxin reductase.

The putative proteins Klglr1p and Kltrr1p can be assigned to the family of FAD-containing pyridine nucleotide:disulfide oxidoreductases.

The amino acid sequence of Klglr1p exhibits strong similarity with other glutathione reductases, as shown in the ClustalW alignment in Fig. 1. Most residues of known catalytic importance (Karimpour *et al.*, 2002; Hirt *et al.*, 2002; López-Barea *et al.*, 1990; Perry *et al.*, 1991; Collinson and Dawes, 1995; Lesk, 2001) are conserved. These are numbered according to the *K. lactis* sequence. The distribution by binding domains is as follows: The N-terminal FAD-binding domain presents two motifs, the glycine-rich motif GXGXXG/A (position 29), involved in binding of coenzymes, and the redox-active disulfide between C-60 and C-65 (CVNVGC) involved in electron flow between NADPH and oxidized glutathione via FAD. This last motif is also present at the FAD-binding domain of mammalian thioredoxin reductases (Karimpour *et al.*,

2002), which are closely related to glutathione reductases (Hirt *et al.*, 2002). The central NADPH-binding domain also contains GXGXXG/A (position 203). In this glycine-rich motif, Y-206 and I-207 are in close contact with the nicotinamide moiety of NADPH, whereas the basic residues R-227 and R-233 interact with the 2´-phosphate group of NADPH and are distinctive of the specific binding of this coenzyme. It has been suggested that A-212 is also involved in the specific binding of NADPH. The C-terminal domain, which forms the interface between subunits in the dimeric proteins, contains a histidine and two glutamate residues (H-473, E-478, E-479) involved in substrate binding. K-69 is also implicated in substrate binding.

```
KlGLR     ----------------------------------MSIRNTINRIIIRRN---------MSD
ScGLR     ----------------------------------MLSATKQTFRSLQIRT---------MST
SpGLR     ------------------------------------------------------------MAP
HsGLR     MALLPRALSAGAGPSWRRAARAFRGFLLLLPEPAALTRALSRAMACRQEPQPQGPPPAAG
                                                                       :

KlGLR     SIRHYDYLVIGGGSGGVASSRRAASYGAKTLLIEAK-AMGGTCVNKGCVPKKVMWYASDL
ScGLR     NTKHYDYLVIGGGSGGVASARRAASYGAKTLLVEAK-ALGGTCVNVGCVPKKVMWYASDL
SpGLR     ISKVFDYLVIGGGSGGLASARRAAKHGAKVALIEASGRLGGTCVNYGCVPKKIMWNIADL
HsGLR     AVASYDYLVIGGGSGGLASARRAAELGARAAVVESH-KLGGTCVNVGCVPKKVMWNTAVH
                :***********:**:****. **:. ::*:   :****** ******:**  :

KlGLR     ATRIGHAHSYNLFEDLPLTKENLTFNWPEFKKKRDAYIHRLNGIYERNLTKEGVDYVYGW
ScGLR     ATRVSHANEYGLYQNLPLDKEHLTFNWPEFKQKRDAYVHRLNGIYQKNLEKEKVDVVFGW
SpGLR     VAKMKTAKQNG-FPNSQLG----SFDWGMIKRKRDAYIGRLNGIYERNVNKDGVAYISGH
HsGLR     SEFMHDHADYG-FPSCEGK-----FNWRVIKEKRDAYVSRLNAIYQNNLTKSHIEIIRGH
            :    . . : .          *:*  :*.*****: ***.**:.*: *. :  : *

KlGLR     ASFTVDGKVQVKKAD-NCTETYTADHILVATGGKPIYP--AKIPGYDYGVSSDEFFELED
ScGLR     ARFNKDGNVEVQKRD-NTTEVYSANHILVATGGKAIFP--ENIPGFELGTDSDGFFRLEE
SpGLR     ASFVSPTEVAVDMNDGSGTQVFSAKYILIAVGGHPIWP--SHIPGAEYGIDSDGFFELES
HsGLR     AAFTSDPKPTIEVSG----KKYTAPHILIATGGMPSTPHESQIPGASLGITSDGFFQLEE
          * *    :  :.  .    : ::* :**:*.** .  *   :*** . *  ** **.**.

KlGLR     QPKKVVVVGAGYIGVEIAGVFNGLGSDSHLVIRGETVLRKFDDCIQETVTDTYIKEGVNI
ScGLR     QPKKVVVVGAGYIGIELAGVFHGLGSETHLVIRGETVLRKFDECIQNTITDHYVKEGINV
SpGLR     QPKRVAIVGAGYIAVELAGVFAALGTETHMFIRQSKFLRKFDPIISDGIMDHFQHIGINV
HsGLR     LPGRSVIVGAGYIAVEMAGILSALGSKTSLMIRHDKVLRSFDSMISTNCTEELENAGVEV
           * : .:******.:*:**:: .**:.: :.** ...**.**  *.    :   : *:::

KlGLR     HKSSNVTKVEKDESTGKLNIQ------LDTGKNIDNVDSLIWTIGRR-SLLGLGLENIGV
ScGLR     HKLSKIVKVEKNVETDKLKIH------MNDSKSIDDVDELIWTIGRK-SHLGMGSENVGI
SpGLR     HTNSLEFKKVEKLPSGELCIH------QQDGSTFN-VDTLLWAIGRAPKIQGLRLEKAGV
HsGLR     LKFSQVKEVKKTLSGLEVSMVTAVPGRLPVMTMIPDVDCLLWAIGRVPNTKDLSLNKLGI
           . *   :  :     :: :           . :  ** *:*:***  .  .:  :: *:

KlGLR     KLDAKEQIVVDEYQNSSVKNVYSLGDVVGKVELTPVAIAAGRKLSNRLFGPEKFKNQKQD
ScGLR     KLNSHDQIIADEYQNTNVPNIYSLGDVVGKVELTPVAIAAGRKLSNRLFGPEKFRNDKLD
SpGLR     KTLPNGIIIADTYQRTNVPTVLSLGDVCGKLELTPVAIAAGRRLSDRLFG--GIKDAHLD
HsGLR     QTDDKGHIIVDEFQNTNVKGIYAVGDVCGKALLTPVAIAAGRKLAHRLFEY--KEDSKLD
          :   :  *:.* :*.:.*  : ::*** **  **********:*:.***     .: : *

KlGLR     YENVPSVVFSHPEAGSIGLSEREAIEKFGKDNVKVYNSKFNAMYYAMMEEKDKTPTRYKL
ScGLR     YENVPSVIFSHPEAGSIGISEKEAIEKYGKENIKVYNSKFTAMYYAMLSE--KSPTRYKI
SpGLR     YEEVPSVVFAHPEAGTIGLTEQEAIDKYGESQIKVYNTKFNGLNYSMVEQEDKVPTTYKL
HsGLR     YNNIPTVVFSHPPIGTVGLTEDEAIHKYGIENVKTYSTSFTPMYHAVTKR--KTKCVMKM
          *:::*:*:*:**  *::*::* ***.*:* .::*.*.:.*. : ::: ..  *     *:

KlGLR     VCTGEEEKVVGLHIIGDSSAEILQGFGVAIKMGATKADFDSCVAIHPTSAEELVTLT
ScGLR     VCAGPNEKVVGLHIVGDSSAEILQGFGVAIKMGATKADFDNCVAIHPTSAEELVTMR
SpGLR     VCAGPLQKVVGLHLVGDFSAEILQGFGVAIKMGATKSDFDSCVAIHPTSAEELVTLV
HsGLR     VCANKEEKVVGIHMQGLGCDEMLQGFAVAVKMGATKADFDNTVAIHPTSSEELVTLR
         **:.  :****:*: *  . *:****.**:******:***. *******:*****:
```

Figure 1: Alignment (ClustalW) of glutathione reductases (GLR) from different organisms. Kl: *K. lactis*; Sc: *S. cerevisiae*; Sp: *Schizosacharomyces pombe*; Ec: *Escherichia coli*; Hs: *Homo sapiens*. Conserved residues involved in catalysis are in bold (see text for details).

The protein Kltrr1p (Fig. 2) belongs to the group of low molecular weight (homodimers, about 35 kDa/subunit) thioredoxin reductases and shows common features with them (Hirt *et al.*, 2002; Pedrajas *et al.*, 1999). At the N-terminal FAD-binding domain, there is a glycine-rich GXGXXG/A motif at position 40. Then, the central NADPH-binding domain contains an active site comprising the redox-active disulfide between C-172 and C-175 (motif CAVC) and a glycine-rich GXGXXG/A motif at position 191. The basic residues R-215 and R-220 that contact the 2´-phosphate group of NADPH are also found. At the C-terminal FAD-binding domain (interface), there are residues 308-309, 313-315 and 317-318, involved in FAD binding, and a glycine-rich GXGXXG/A motif at position 332. A fourth binding domain containing a selenocysteine residue atthe penultimate position and typical of higher molecular weight mammalian thioredoxin reductases (Hirt *et al.*, 2002) is absent in *Kl*Trr1p.

```
KlTRR1    MLLVRNSTLGRLSSLRGFFRNINESNIFYRMVHHKVTIIGSGPAAHTAAIYLARAEIKPT
ScTRR1    ----------------------------MVHNKVTIIGSGPAAHTAAIYLARAEIKPI
ScTRR2    --MIKH----IVSPFRTNFVGISKS-VLSRMIHHKVTIIGSGPAAHTAAIYLARAEMKPT
SpTRR     ----------------------------MTHNKVVIIGSGPAGHTAAIYLARGELKPV
                                       * *:**.*******.*********.*:**

KlTRR1    LYEGFMANGIAAGGQLTTTTEIENFPGFPDGLTGSELMDRMKAQSIKFGTDVITETVSKV
ScTRR1    LYEGMMANGIAAGGQLTTTTEIENFPGFPDGLTGSELMDRMREQSTKFGTEIITETVSKV
ScTRR2    LYEGMMANGIAAGGQLTTTTDIENFPGFPESLSGSELMERMRKQSAKFGTNIITETVSKV
SpTRR     MYEGMLANGIAAGGQLTTTTDVENFPGFPDGINGTTLTENFRAQSLRFGTEIITETVSKL
          :***::***************::*******:.:.*: * :.:: ** :***::*******:

KlTRR1    DLSSRPFKFWTEFNEDQEPETTDAIILSTGASAKRLHLPGEETYWQQGISACAVCDGAVP
ScTRR1    DLSSKPFKLWTEFNEDAEPVTTDAIILATGASAKRMHLPGEETYWQKGISACAVCDGAVP
ScTRR2    DLSSKPFRLWTEFNEDAEPVTTDAIILATGASAKRMHLPGEETYWQQGISACAVCDGAVP
SpTRR     DLSSRPFKYWLEGAEEEEPHTADSVILATGASARRLHITGEDTYWQAGISACAVCDGAVP
          ****:**: * *  *: ** *:*::**:*****:*:*:.**:**** ************

KlTRR1    IFRNKPLAVIGGGDSACEEAQFLTKYGSKVYMLVRKDHLRASQIMQRRAEQNEKIEILYN
ScTRR1    IFRNKPLAVIGGGDSACEEAQFLTKYGSKVFMLVRKDHLRASTIMQKRAEKNEKIEILYN
ScTRR2    IFRNKPLAVIGGGDSACEEAEFLTKYASKVYILVRKDHFRASVIMQRRIEKNPNIIVLFN
SpTRR     IYRNKPLAVVGGGDSAAEEAQFLTKYGSKVYVLVRRDKLRASPIMAKRLLANPKVEVLWN
          *:*******:******.***:*****.***::***:*::*** ** :*   * :: :*:*

KlTRR1    HVTLEAKGDGKYLNALKVKNVKTNEEYDLPVNGLFYAIGHTPATNIVAGQVDLDEAGYVK
ScTRR1    TVALEAKGDGKLLNALRIKNTKKNEETDLPVSGLFYAIGHTPATKIVAGQVDTDEAGYIK
ScTRR2    TVALEAKGDGKLLNMLRIKNTKSNVENDLEVNGLFYAIGHSPATDIVKGQVDEEETGYIK
SpTRR     TVAEEAQGDGKLLNNLRIKNTNTNEVSDLQVNGLFYAIGHIPATKLVAEQIELDEAGYIK
           *: **:**** ** *::**.:.*   ** *.******** ***.:*  *:: :*:**:*

KlTRR1    TVPGSTLTNVPGVFAAGDVQDARYRQAITSAGSGCMAALDAEKYITELE---
ScTRR1    TVPGSSLTSVPGFFAAGDVQDSKYRQAITSAGSGCMAALDAEKYLTSLE---
ScTRR2    TVPGSSLTSVPGFFAAGDVQDSRYRQAVTSAGSGCIAALDAERYLSAQE---
SpTRR     TINGTPRTSIPGFFAAGDVQDKVFRQAITSAGSGCQAALLAMHYLEELEDTD
          *: *:. *.:**.******** :***:******* *** * :*:   *
```

Figure 2: Alignment (ClustalW) of thioredoxin reductases (TRR) from different organisms. Kl: *K. lactis*; Sc: *S. cerevisiae* (TRR1 is the cytosolic isoform and TRR2 is the mitochondrial isoform); Sp: *Schizosacharomyces pombe*; Ec: *Escherichia coli*. Conserved residues involved in catalysis are in bold (see text for details). The predicted mitochondrial-targeting signal is underlined.

Kltrr1p shows a mitochondrial-targeting signal of 29 residues, as predicted by MitoProt II (Claros and Vicens, 1996) with a probability of 0.99. Using the information about *K. lactis* sequences recently available from Genolevures (http://cbi.labri.u-bordeaux.fr/Genolevures/), we have verified the absence of thioredoxin reductase isoforms; in fact, the protein with the highest similarity to Kltrr1p shows only 24% identities (M. Bolotin-Fukuhara, personal communication) which argues in favor of Kltrr1p being the unique *K. lactis* thioredoxin reductase. This contrasts with the situation in *S. cerevisiae* where two isoforms are present, Trr1p (cytosolic) and Trr2p (mitochondrial) (Grant, 2001).

The response of these glutation and thioredoxin reductase systems to the oxidative stress created by exogenous addition of H_2O_2 was studied both by measuring mRNA levels and enzyme activities.

For these assays, the yeast cells were grown in Erlenmeyer flasks at 30°C and 250 rpm in synthetic complete medium (CM) containing either 2% glucose or 2% fructose (supplemented with 0.05% glucose for the *rag2* strain) up to OD_{600}=0.6 and shifted for 15 or 30 minutes (RNA or protein extraction, respectively) to CM+H_2O_2. To generate oxidative stress conditions, hydrogen peroxide was added to a final concentration of 0.4 mM as previously reported (Billard *et al.*, 1997).

Two different *K. lactis* strains were used in these experiments, the wild type NRRL-Y1140 (*MATa*, ATCC8585, CBS2359) and the *rag2* (phosphoglucose isomerase) mutant PM5-2D (*MATa uraA1-1 metA1-1 argA1-1 trpA1-1 rag2-1*). The *rag2* strain was selected for two main reasons. First, the *K. lactis rag2* mutant growing in glucose shows similar carbohydrate metabolic flux to *S. cerevisiae* under oxidative stress, that is, glucose is redirected to the pentose phosphate pathway at the expense of glycolysis (Godon *et al.*, 1998; González Siso *et al.*, 1996a) and, therefore, a higher intracellular turnover of NADPH, which provides reducing power to reductases of glutathione and thioredoxin, is predicted. Second, when the *K. lactis* phosphoglucose isomerase mutant grows in glucose, the activity of the respiratory chain increases (González Siso *et al.*, 1996a); the concomitant increase in ROS generation (Cabiscol *et al.*, 2000) could induce the oxidative stress response.

Northern blot analysis was performed as described earlier (González-Dominguez *et al.*, 2000); hybridization was quantified using a 425 PhosphorImager Scanning Instrument and the ImageQuant programme (Molecular Dynamics). Data were normalized for RNA loading against the signal obtained from the control probe *rRNA 25S*. Enzymatic activities were measured as explained above.

Fig. 3A shows distinct responses of *KlGLR1* and *KlTRR1* to H_2O_2 treatment. In the conditions assayed in this work, *KlTRR1* mRNA is detected only after treatment

with H_2O_2 while *KlGLR1* mRNA level is not affected by addition of H_2O_2. In accordance with this result, a consensus for putative binding of Yap1p (ATGAATCAG) is found in the *KlTRR1* promoter region (position –223 to –231) but not in the *KlGLR1* promoter (search performed with TFSEARCH through www.cbrc.jp/htbin/nph-tfsearch). This result contrasts with the hitherto described behaviour of the homologous *S. cerevisiae* genes since *TRR1/TRR2* but also *GLR1* are Yap1p targets induced by H_2O_2 (Pedrajas *et al.*, 1999; Grant *et al.*, 1996a; Grant *et al.*, 1996b; Ross *et al.*, 2000). We have verified that the transcriptional response of *KlGLR1* and *KlTRR1* to exogenously added H_2O_2 is not strain-dependent since there were no significant differences between the wild type and the *rag2* strains. Furthermore, no significant differences in this response were observed for both strains growing in glucose or fructose in spite of the predicted different intracellular NADPH turnover occurring in each case, according to the carbon source utilization.

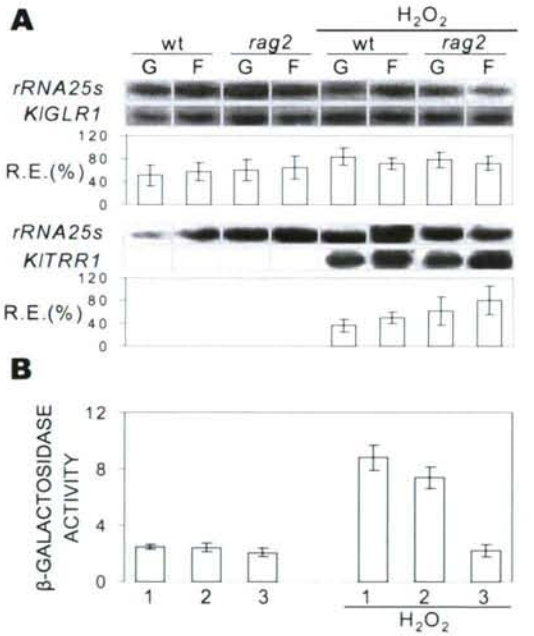

Figure 3: (A) Effect of H_2O_2 exposure on mRNA levels of *KlGLR1* and *KlTRR1* in wild type (wt) and phosphoglucose isomerase mutant (*rag2*) *K. lactis* strains growing in glucose (G) or fructose (F). R.E. = Relative Expression. After loading normalization, the highest intensity signal in each blot was considered to have an R.E. value of 100% and was used as the reference for the other signals. The Northern blots shown are representative of two independent RNA extractions assayed in duplicate. Therefore, values of R.E. are the mean ± standard deviation of four signals. (B) Effect of H_2O_2 exposure on β-galactosidase activities shown by the *lacZ* fusions to the following *KlTRR1* serial promoter deletions: (1) from nucleotide -1005 to the ATG, (2) from -286, (3) from -199. The Yap1p binding site is located from position -231 to -223. Enzyme Units were calculated as described in (Adams *et al.*, 1997). Values represent the mean ± standard deviation of six measurements, corresponding to three individual transformed colonies assayed in duplicate.

The same conclusions could be inferred from the determination of enzyme activities (Table 1). There is a significant increase in thioredoxin reductase activity after H_2O_2 treatment, glutathione reductase activity being unaffected in both strains and with independence of the carbon source used for growth.

Table I. Specific glutathione reductase and thioredoxin reductase activities (E.U., mean ± standard deviation, two duplicated independent experiments, i.e., n=4) in response to treatment with hydrogen peroxide in the *K. lactis* strains Y1140 (wild type) and PM5-2D (phosphoglucose isomerase mutant) growing in glucose and fructose media.

		Glutathione reductase		**Thioredoxin reductase**	
		-H_2O_2	**+H_2O_2**	**-H_2O_2**	**+H_2O_2**
Y1140	**Glucose**	0.09 ± 0.014	0.11 ± 0.012	0.07 ± 0.003	0.14 ± 0.005
	Fructose	0.10 ± 0.005	0.09 ± 0.008	0.06 ± 0.003	0.14 ± 0.016
PM5-2D	**Glucose**	0.14 ± 0.009	0.14 ± 0.008	0.08 ± 0.006	0.15 ± 0.004
	Fructose	0.09 ± 0.002	0.13 ± 0.009	0.12 ± 0.010	0.24 ± 0.016

For conditions see text.

To study the functionality of the putative Yap1p binding site found in the *KlTRR1* promoter, three unidirectional deletions were constructed by PCR amplification of different size *KlTRR1* promoter fragments (from nucleotides -1005 to +3, -286 to +3 and -199 to +3) and cloning the amplified products in the *Bam*HI-*Hind*III sites of pXW2 (Chen *et al.*, 1992) fused in frame to the eighth codon of the *lacZ* reporter gene. The correctness of the resultant constructions was verified by sequencing. These *lacZ* fusions and the empty vector were used to transform by electroporation the β-galactosidase deficient *K. lactis* strain MW190-9B (*MATa lac4-8 uraA Rag*$^+$). For quantification of the β-galactosidase activity, three independent transformed colonies were picked randomly, grown in CM-ura up to OD_{600}=0.6 and shifted for 15 minutes to the same medium with 0.4 mM H_2O_2. β-Galactosidase assays were performed in duplicate with permeabilized cells as described in Adams *et al.* (1997).

Fig. 3B shows that in the two fusions containing the Yap1p binding site, β-galactosidase activity increased significantly, and up to a similar level, after H_2O_2 treatment. When the Yap1p binding site is removed from the promoter, the activity is similar with and without H_2O_2 treatment. Therefore, the induction of *KlTRR1* transcription by H_2O_2 is mediated through this Yap1p binding site.

In conclusion, of the two genes (*KlGLR1* and *KlTRR1*) isolated and characterized in this work, only *KlTRR1* is regulated in response to an oxidative stress created by exposure of *K. lactis* cells to H_2O_2. *KlTRR1* mRNA levels and thioredoxin

reductase activity increase after H_2O_2 treatment but *KlGLR1* mRNA levels and glutathione reductase activity remain unaffected. These results suggest that the oxidative stress response in the respiratory yeast *K. lactis* does not follow exactly the same pattern described for the fermentative yeast *S. cerevisiae*. A genome-wide strategy to compare transcriptional responses of these two yeasts under oxidative stress conditions could be very valuable in the near future.

Acknowledgements

PM5-2D and MW190-9B strains were kindly provided by Dr Wésolowski-Louvel (Université Claude Bernard, Lyon, France). Manuel Becerra helped us with protein purification. S.D.P. was the recipient of a fellowship from the Xunta de Galicia (Spain), N.T. was the recipient of a fellowship (FPU) from the Ministerio de Educación (Spain). This work was funded by grants PGIDT00PXI10303PR from the Xunta de Galicia (Spain), BMC2000-0117 and BMC2003-04992 from the Ministerio de Ciencia y Tecnología (Spain).

CHAPTER 3

Genome-wide analysis of *Kluyveromyces lactis* in wild-type and *rag2* mutant strains

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SUMMARY

The use of heterologous DNA-arrays from *Saccharomyces cerevisiae* has been tested and revealed as a suitable tool to compare the transcriptomes of *S. cerevisiae* and *Kluyveromyces lactis*, two yeasts with notable differences in their respiro-fermentative metabolism. The arrays have also been applied to study the changes in the *K. lactis* transcriptome owing to mutation in the *RAG2* gene coding for the glycolytic enzyme phosphoglucose isomerase. Comparison of the *rag2* mutant growing in 2% glucose versus 2% fructose has been used as a model to elucidate the importance of transcriptional regulation of metabolic routes, which may be used to reoxidize the NADPH produced in the pentose phosphate pathway. At this transcriptional level, routes related to the oxidative stress response become an interesting alternative for NADPH use.

INTRODUCTION

Kluyveromyces lactis has become an alternative model to the traditional yeast *Saccharomyces cerevisiae* owing to its industrial applications and metabolic peculiarities. Both yeasts belong to the facultative aerobic group that displays the ability to metabolize glucose by oxidative and oxidoreductive pathways. The difference lies in the relative importance of these routes in aerobic conditions; *S. cerevisiae* is an aerobic fermentative yeast and *K. lactis* is an aerobic respiratory yeast (reviewed in González Siso *et al.*, 2000). Aerobic fermentation, under fully-aerobic conditions is known as the Crabtree effect and *S. cerevisiae* is the prototype of a Crabtree-positive yeast (Rieger *et al.*, 1983). *K. lactis* has been reported as Crabtree-negative in fully oxidative conditions (González Siso *et al.*, 1996b; Kiers *et al.*, 1998).

Several strains of *K. lactis* are Rag^+ (resistance to antimycin A in glucose), and therefore, they are able to grow on glucose in presence of antimycin A due to their ability to ferment glucose to ethanol when respiration is impaired. Rag^- strains of *K. lactis* are naturally occurring mutants in which glycolysis or fermentation is not possible; however, these strains are able to grow on glucose, revealing that the sugar can be oxidized to C3 intermediates using the pentose phosphate pathway and further oxidized to CO_2 if the respiratory chain is not blocked. The RAG- phenotype has led to the cloning and characterization of several *K. lactis* genes encoding glucose transporters, glycolytic and fermentative enzymes or proteins implied in their transcriptional regulation (reviewed in Breunig *et al.*, 2000). The *K. lactis* mutants from *RAG2*, encoding the glycolytic enzyme phosphoglucose isomerase, grow in glucose

(Goffrini *et al.*, 1991). On the contrary, phosphoglucose isomerase mutants of S. *cerevisiae* (*Δpgi*) cannot grow on glucose as the sole carbon source (Maitra, 1971), suggesting that the pentose phosphate pathway in *S. cerevisiae* does not contribute substantially to glucose metabolism.

The sequencing of the whole genome of the yeast *S. cerevisiae* enables genome-wide analysis of gene expression. The genomic techniques first developed for *S. cerevisiae* are now being applied to other genomes. Since DNA arrays of the whole genome are still not available for *K. lactis*, in this report, we describe a genome-wide survey of *K. lactis* transcriptome using arrays from the closely related yeast *S. cerevisiae*. Partial sequencing of the *K. lactis* genome (Ozier-Kalogeropoulos *et al.*, 1998; Llorente *et al.*, 2000) reveals that the homology between the genes of these two yeasts is good enough to allow heterologous hybridization. To verify the validity of this heterologous array system, we performed a comparative analysis of RNA levels in *S. cerevisiae* and *K. lactis* cells grown in glucose. Data are in accordance with previous results about metabolic differences between these yeasts. In addition, we have analyzed the *K. lactis* transcriptome in wild type (wt) and *rag2* mutant strains growing in 2% fructose or 2% glucose as a model to investigate the possible routes to reoxidize the NADPH produced by the pentose phosphate pathway.

MATERIALS AND METHODS

Strains and media

Cells from the *S. cerevisiae* aGH1 (*MAT***a** *trp*1-289 *leu*2-3 *leu*2-112 *gal*1Δ152) and the *K. lactis* strains NRRL-Y1140 (CBS2359) and PM5-2D (*MAT***a** *ura*A1-1 *met*A1-1 *arg*A1-1 *trp*A1-1 *rag*2-1) were grown at 30°C to OD_{600} 0.8 and collected by centrifugation. The strains aGH1 and NRRL-Y1140 were grown in complete media with glucose (Zitomer and Hall, 1976) and the mutant strain PM5-2D with 2% glucose or 2% fructose as required. The *rag*2 mutant was supplemented with 0.05% glucose to facilitate growth.

DNA arrays

The use of arrays was as previously described (Becerra *et al.*, 2002). For RNA isolations, cells were harvested and immediately frozen in liquid nitrogen. Pellets were disrupted with a Micro-Dismembrator (B. Braun Biotech International). The resulting powder was mixed with TRIZOL Reagent (Life Technologies) and total RNA was extracted by the method of Chomczynski and Sacchi (1987). Probe generation was as described in Hauser *et al.*, (1998). Briefly, 60 μg of total RNA was annealed to oligonucleotide dT_{15} and used as a template to synthesize and radiolabel the corresponding first strand cDNA with 50 μCi (1μCi = 37kBq) of [α-^{33}P]-dCTP (Amersham) and SuperScript II (Life Technologies). The reactions were carried out at 43°C for 1 h, after which the RNA was hydrolysed with NaOH at 65°C for 30 minutes. The probe was purified by isopropanol precipitation and isotope incorporation was measured to check the efficiency of the reaction. Filters were pre-hybridized for 1 hour at 65°C in the hybridization mix: 5x SSC, 5x Denhardt's solution and 0.5% SDS. The probe was then denatured for 5 minutes at 100°C, cooled quickly on ice and hybridized with the arrays overnight at 65°C. The next day, two washes were carried out, at hybridization temperature, for 5 and 20 minutes respectively, in 2x SSC and 0.1% SDS. Filter regeneration was done by pouring a boiling solution of 5mM sodium phosphate (pH 7.5) and 0.1% SDS over the filters prior to their reuse. The filters were exposed for 24 hours to a storage phosphor screen and data collected

using a PhosphorImager Scanning Instrument 425 (Molecular Dynamics). Signal quantification was performed with Array Vision software (Molecular Dynamics) that localizes over each array element a bounding circle fitted to the size of the DNA spot. At least two replica spots per gene and condition were analyzed. The data were normalized, processed, clustered and analyzed using the informatics program GENESPRING (Silicon Genetics). Normalization of data from each membrane was carried out by dividing the measurement for each gene by the 50th percentile of all measurements in this membrane. The bottom 10th percentile was used as a test for correct background subtraction. To compare the expression of each gene in the different conditions, all the measurements for that gene were divided by the median of the gene's expression values over all the samples. The complete list for all genes as well as the mean of signal intensities is available as supplementary data accompanying this paper.

Other methods

Glycogen storage in yeast cells was examined by a plate assay in which cells are stained with iodine as previously reported (Rowen *et al.* 1992).

Northern blot analysis was performed as described earlier (González-Domínguez *et al.* 2000).

For the determination of enzyme activities, protein extracts were prepared as follows. Cultures were grown in Erlenmeyer flasks at 30°C and 250 rpm in the selected medium up to OD_{600}=0.6. The cells from 400 mL of culture were collected by centrifugation and resuspended in 1 mL of buffer A (0.2 M Tris-HCl pH=7.8, 0.3 M $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 2mM β-mercaptoethanol and a protease inhibitor cocktail) per gram wet mass. Cells were broken by vortexing with glass beads in 20 second pulses. The supernatant of a centrifugation at 8000 rpm for 15 minutes was immediately used. All steps were performed in cold. Protein concentration was measured by the method of Bradford (1976) using bovine seroalbumin as a standard. Glutamate dehydrogenase activity was determined by the method of Doherty (1970). Enzyme Units (E.U.) are expressed in terms of micromoles of NADPH oxidized per milligram of protein per minute, in the assay conditions. Glucose 6-P-dehydrogenase activity was determined by the method of Kuby and Noltmann (1966). Enzyme Units are expressed in terms of micromoles of NADPH formed per milligram of protein per minute, in the assay conditions.

Data are expressed as means ± standard deviation (S.D.). ANOVA and Multiple Comparisons Tests, using Statgraphics Plus software, evaluated the statistical significance of differences found between means at the 95% confidence level.

RESULTS AND DISCUSSION

The transcriptome of *K. lactis* versus *S. cerevisiae*

The *K. lactis* genome has been estimated in a total amount of 12 Mbp distributed in VI chromosomes (Ozier-Kalogeropoulos *et al.*, 1998). Using data from partial sequencing, comparison with the completed *S. cerevisiae* genome revealed a mean of approximately 63% in amino acid identity between the two yeasts (Bolotin-Fukuhara *et al.*, 2000). Taking into account that DNA-arrays from *K. lactis* are not yet available, the close relationship between their genomes suggested to us the use of heterologous DNA-arrays from *Saccharomyces cerevisiae* as a suitable tool to compare the transcriptomes of *S. cerevisiae* and *K. lactis*.

We have analyzed the expression of the *S. cerevisiae* and *K. lactis* yeast genomes in complete media with glucose as carbon source. Transcriptomes were screened using the high-density membrane hybridization method (Hauser *et al.*, 1998). RNA was extracted from the cultures of aGH1 and NRRL-Y1140. The labeled cDNAs

obtained from each RNA sample were used to probe the DNA arrays containing the whole set of genes from *S. cerevisiae* (Hauser *et al.*, 1998). Data were analyzed by statistical procedures using the program GENESPRING (Silicon Genetics) as described in Material and Methods.

Fig. 1A shows the result of the comparative analysis performed. The genes that are over the median of normalized values (>1) between the different samples are in red and those below in blue (<1). The colour distribution in the two parts of the figure clearly shows that there is a difference in the distribution of signal intensities obtained in the two arrays. Some genes that show strong expression in *S. cerevisiae* are expressed at low levels in *K. lactis* and vice versa. Despite the lower intensity obtained, attributable to the lowest homology between the cDNA and the probes, a signal is obtained for the 94% of the heterologous probes. Fig. 2A shows in more detail the distribution of normalized levels of expression in the two yeasts. Although there are more genes with the lowest levels of expression in *K. lactis* (1244 genes <0.5 in *K. lactis* versus 515 genes <0.5 in *S. cerevisiae*) the number of genes with medium or high level of expression is enough to have a general picture of the transcriptome in this yeast. Using *K. lactis* partial sequences, available at Genolevures (http://cbi.labri.u-bordeaux.fr/Genolevures/), we have verified that there is no statistical correlation between the homology of the translated open reading frames (ORFs) in the two yeasts and the intensity of the signal in the *K. lactis* transcriptome. Two pools of 50 ORFs each were selected; the first including ORFs producing the highest signals in the array and the second the ORFs with the lowest signals. Correlation between percent of identity, between *S. cerevisiae* and *K. lactis* translated ORFs, and the intensity of the array signals was below 0.1 in the two pools. Fig. 2B shows the values of minimum, maximum and average percent of identity in the pools of high and low expression. The distribution of the number of ORFs in different ranges of identity is very similar in the two pools (Fig. 2C). These data indicate that differences in the transcriptomes are not attributable merely to differential hybridization with homologous or heterologous probes.

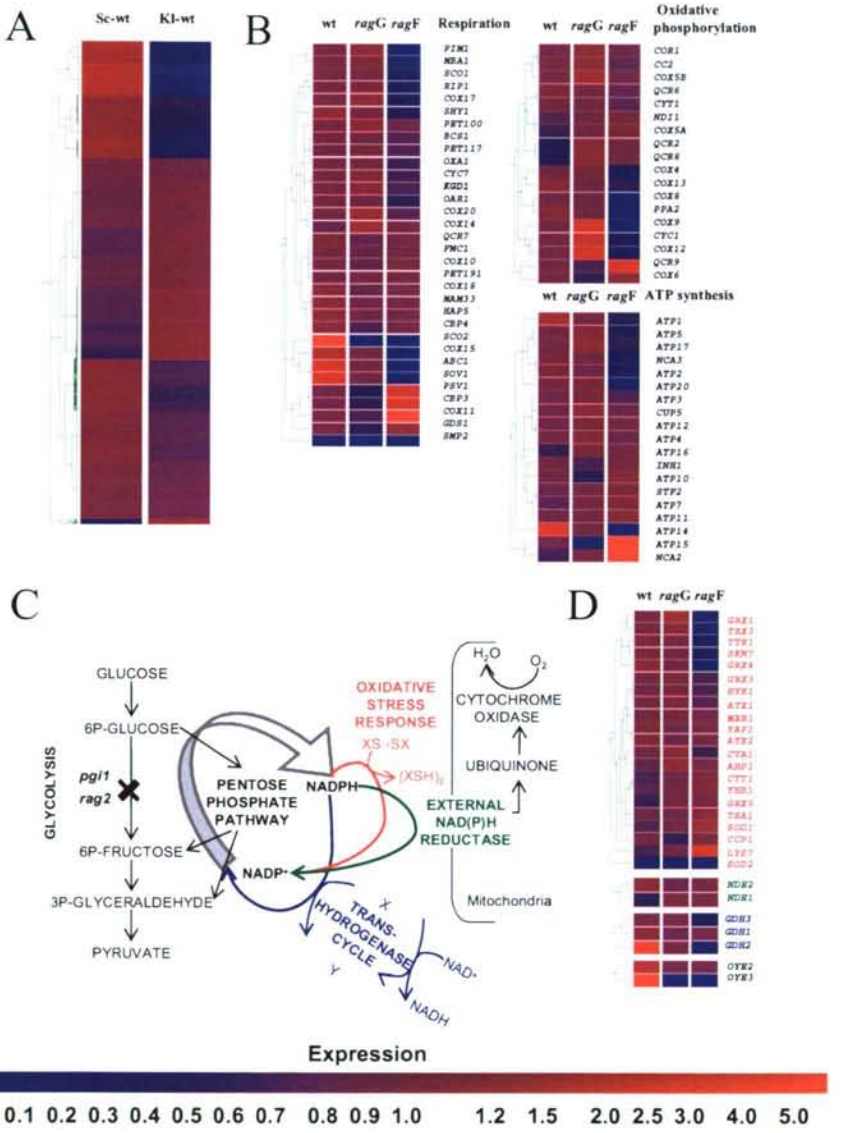

Figure 1: Comparative cluster analysis of the transcriptomes. The genes that are over the median of normalized values between the different samples are in red (>1) and those below in blue. The relationship between the intensity of the colour is proportional to the normalized values as represented on the scale at the bottom of the figure (A) The whole transcriptome of wild type strains of *S. cerevisiae* versus *K. lactis* in glucose medium. (B) Clusters of genes belonging to the functional groups of ATP synthesis, oxidative phosphorylation and respiration from the wild type in glucose (*wt*) and *rag*2 mutant strains of *K. lactis* in glucose (*rag*G) and fructose (*rag*F) media. (C) Scheme of putative pathways for cytosolic NADPH reoxidation as discussed in the text (D) Array data from the wt and *rag*2 mutant strains in glucose (*rag*G) and fructose (*rag*F) media for the genes related to pathways outlined in Fig. 1C; The colour code of gene names in Fig. 1D of the figure is as for the colour code assigned in Fig. 1C, i.e., mitochondrial external dehydrogenases (*NDE*1 and *NDE*2), glutamate dehydrogenase (*GDH*1, *GDH*2 and *GDH*3), functional group of oxidative stress response and old yellow enzyme (*OYE*2 and *OYE*3).

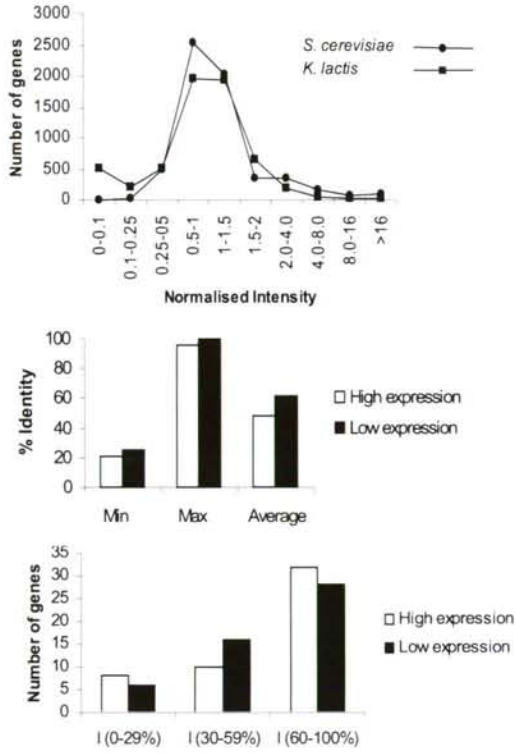

Figure 2: Evaluation of the use of *S. cerevisiae* DNA arrays to study the *K. lactis* transcriptome. (A) Number of genes of the wild type strains of *K. lactis* and *S. cerevisiae* as a function of the normalized expression levels obtained in the arrays. (B) Values of minimum, maximum and average percent of identity between translated ORFs from *S. cerevisiae* and *K. lactis* in the pools of high and low expression *K. lactis* genes. (C) Distribution of the number of ORFs in different ranges of identity in the two pools.

The correlation coefficient (CC) between the whole set of data obtained using cDNA from *S. cerevisiae* or *K. lactis* is moderate to low (0.335), however, analyzing the correlation between groups of genes clustered by functional distribution described by MIPS (http://mips.gsf.de/genre/proj/yeast/index.jsp) it is possible to find out that the expression of some clusters of genes is highly correlated while others are poorly correlated. Table 1 summarises the CC values found for several representative groups. Some functional groups related to housekeeping functions such as mitosis, transcription, cell wall biogenesis and others described in the upper part of Table 1 are highly correlated. On the other hand, at the bottom of the table and with the lowest CC, there are several groups of genes whose expression is not related in the two yeasts. The CC for the genes related to pentose phosphate pathway is reasonable good because the relative pattern of expression is similar in the two yeasts, although *K. lactis* uses the pentose phosphate pathway more efficiently as previously described (Jacoby

et al., 1993). Taking into account that *K. lactis* and *S. cerevisiae* differ in their respiro-fermentative metabolisms (González Siso *et al.*, 2000) it is interesting that the highest differences are found between the two transcriptomes in groups of genes that are related to carbohydrate metabolism and respiratory functions. This validates the use of heterologous arrays from *S. cerevisiae* to analyze the transcriptome of *K. lactis* under different conditions or genetic backgrounds. Other functional *S. cerevisiae* categories with low CC in the table have been cited in the literature as differentially regulated in *K. lactis* versus *S. cerevisiae*; this is the case of silencing (Åström and Rine, 1998) or histidine metabolism (Lamas-Maceiras *et al.*, 1999). The low level of correlation between genes related to recombination could explain why it is difficult to knock out genes in *K. lactis* by homologous recombination (Wésolowski-Louvel *et al.*, 1996).

Table I: Correlation coefficients (CC) between RNA levels of functional groups of genes from *S. cerevisiae* (aGH1) and *K. lactis* (Y1140) strains in 2% glucose (calculated from crude data).

Functional group	*N*	CC aGH1/Y1140
mRNA 3'-end processing	11	0.974
mRNA processing	16	0.968
Cell cycle	95	0.957
Transcription	201	0.935
Protein degradation	107	0.934
Mitosis	68	0.895
Endocytosis	19	0.890
Cell wall biogenesis	71	0.885
tRNA processing	22	0.849
Mating	39	0.799
Pentose phosphate cycle	9	0.772
Fatty acid metabolism	24	0.766
Sporulaiton	33	0.753
Heme biosynthesis	8	0.709
Protein glycosylation	55	0.702
Phospholipid metabolism	25	0.699
Signalling	51	0.664
Cytoskeletal organisation	92	0.611
Silencing	26	0.525
ATP synthesis	19	0.428
Glycolysis	38	0.312
Respiration	31	0.298
Oxidative phosphorylation	18	0.247
TCA cycle	23	0.206
Transport	147	0.170
Sterol metabolism	31	0.152
Mitochondrial related	47	0.126
Meiosis, recombination	11	0.115
Drug resistance	16	0.077
Oxidative stress response	20	-0.309

Note: *N*, number of genes.

The transcriptome of the wild type and *rag2* mutant strains from *K. lactis* growing in glucose or fructose

In the first part of this work we have shown the validity of the use of heterologous arrays from *S. cerevisiae* to analyze the transcriptome of *K. lactis* and also that some differences in the respiro-fermentative metabolism of both yeasts may be inferred from the comparison of their transcriptomes. With this premise, we also analyzed the genome expression of *K. lactis* NRRL-Y1140 wild type in 2% glucose and a *rag2* mutant in complete medium with 2% glucose or fructose and we compared the CC between key groups of genes directly related to carbohydrate metabolism and respiratory functions to detect the possible metabolic disturbances caused by the mutation in the phosphoglucose isomerase step (Table 2). We observe that in all of the groups analyzed the CC is high (>0.87) between the wild type and the *rag2* mutant strains growing in glucose, but low for the comparison between the *rag2* mutant growing in glucose vs. fructose, with the exception of glycolysis which correlates very well in both cases. The significance of these differences in the CC values was validated experimentally for the group of genes of glycogen metabolism. The low CC values for the comparison of *rag2* in glucose versus fructose are actually related to differences in the accumulation of glycogen as shown in Fig. 3.

Table II: Correlation coefficients (CC) between RNA levels of functional groups of genes from wild type and *rag2 K. lactis* strains in 2% glucose (G) or 2% fructose (F) (calculated from crude data).

Functional group	*N*	CC *rag2*/Y1140	CC *rag2* G/F
ATP synthesis	19	0.879	0.321
Glycogen metabolism	8	0.976	0.390
Glycolysis	38	0.998	0.973
Oxidative phosphorylation	18	0.868	0.685
Pentose phosphate cycle	9	0.951	0.717
Respiration	31	0.895	0.427
TCA cycle	23	0.869	0.624

Note: *N*, number of genes

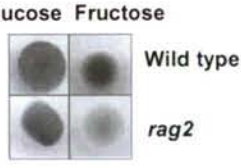

Figure 3: Effect of the *rag2* mutation on glycogen storage by *K. lactis*. Glycogen storage in yeast cells was examined by a plate assay in which cells are stained with iodine as previously reported (Rowen *et al.* 1992)

In conclusion, data obtained show that the mutation in the phosphoglucose isomerase causes in *K. lactis* very little disturbance upon transcription of genes related to pentose phosphate pathway and glycolysis when glucose is the carbon source, which may be due to the fact that this yeast, even wild type strains, preferentially use the pentose phosphate pathway to catabolise glucose (Jacoby *et al.*, 1993). We corroborated this conclusion by studying in more detail the mRNA levels and enzymatic activity of the key enzyme glucose 6-phosphate dehydrogenase that catalyzes the first irreversible reaction of the pentose phosphate pathway, where NADPH is produced. In Fig. 4A a northern analysis using a *K. lactis* glucose 6-phosphate dehydrogenase probe (*K. lactis ZWF1* gene accession No. X70373) is shown. The mRNA levels of glucose 6-phosphate dehydrogenase in the *K. lactis rag2* mutant grown in glucose versus fructose are similar. Moreover, to rule out a posttranscriptional regulation, glucose 6-phosphate dehydrogenase activity was measured.

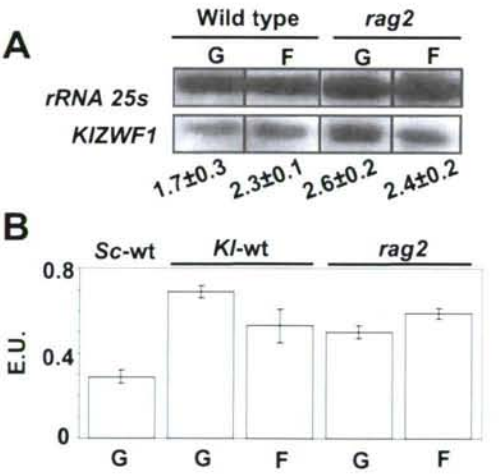

Figure 4: (A) Northern blot analysis of the *KlZWF1* gene expression from the wild type (wt) and *rag2* strains of *K. lactis* in glucose (G) and fructose (F) media. n=2. Normalized values of expression are calculated as the quotient of the signals *KlZWF1/rRNA25S* multiplied by 100. Densitometric raw data *KlZWF1/rRNA25S* are as follows; experiment 1, wt G 197/13847, wt F 1146/46259, *rag2* G 1020/42861, *rag2* F 876/32122; experiment 2, wt G 905/43920, wt F 946/41313, *rag2* G 557/19312, *rag2* F 674/30305 (B) glucose 6-phosphate dehydrogenase activity in the same conditions and in the *S. cerevisiae* wild type strain in glucose medium. n=5

In Fig. 4B we observe that the glucose 6-phosphate dehydrogenase activity is significantly higher in *K. lactis* than in *S. cerevisiae*, which agrees with previous reports of higher glucose 6-phosphate dehydrogenase activity in Crabtree-negative versus Crabtree-positive yeasts (Zeeman *et al.*, 2000). Importantly, there is no increase in glucose 6-phosphate dehydrogenase activity for the *K. lactis rag2* mutant growing in

glucose versus fructose. A change in the carbon source from glucose to fructose in the *rag2* mutant provokes a disturbance in several metabolic pathways varying mRNA levels, as can be deduced from the CC shown in Table 2. It seems obvious that differences in intracellular NADPH turnover are occurring since in the *K. lactis rag2* mutant all of the glucose used as substrate must be necessarily metabolized through the pentose phosphate pathway (oxidative and non-oxidative) thus yielding a high NADPH amount that must be reoxidized, whereas fructose may enter directly into glycolysis, as fructose 6-phosphate, or into the non-oxidative part of the pentose phosphate pathway (Schaaff *et al.* 1990), but without NADPH production.

The transcriptome of rag2 in glucose* versus *fructose, a model to investigate NADPH reoxidation

Clustering analysis of gene expression applied to functional groups related to oxidative metabolism like ATP synthesis, oxidative phosphorylation or respiration shows that the metabolism of the *rag2* mutant in fructose is likely less respiratory and energy generating than in glucose (Fig. 1B). This is in agreement with transcriptional expression and oxygen consumption data previously published by our group (González Siso *et al.*, 1996a) and is also supported by the fact that the growth rate of the rag2 mutant in glucose is higher than in fructose (glucose = 0.287 ± 0.026 h^{-1}. n=3; fructose = 0.105 ± 0.009 h^{-1}. n=3).

We have used the *K. lactis* phosphoglucose isomerase *rag2* mutant (Goffrini *et al.*, 1989) as a tool to investigate NADPH reoxidation, since in this mutant all the glucose must be necessarily re-routed through the pentose phosphate pathway to bypass the blocked glycolytic step. Fig. 1C shows a scheme of the pathways that have been considered as putatively implicated in NADPH reoxidation in the *K. lactis rag2* mutant growing in glucose, and whose role we analyzed in reference to data obtained from the arrays.

The fact that the growth in glucose of the *K. lactis rag2* mutant is prevented by a blockade of the mitochondrial respiratory chain after ubiquinone (Goffrini *et al.*, 1991), together with our observations, suggested that in *K. lactis* NADPH reoxidation could be mediated by a cytoplasmic side mitochondrial NAD(P)H dehydrogenase that would pass the electrons to ubiquinone (González Siso *et al.*, 1996a). Consistent with this hypothesis, mitochondria of *K. lactis* can oxidize cytosolic NADPH (Overkamp *et al.*, 2002) whereas *S. cerevisiae* mitochondria cannot (Small and McAlister-Henn, 1998; Luttik *et al.*, 1998). Differences in the expression levels of external mitochondrial dehydrogenases between the *rag2* strain growing in glucose or fructose are not observed (Fig. 1D). Therefore, differences in NADPH reoxidation between *K. lactis* and

S. cerevisae are not attributable to transcriptional regulation of external mitochondrial dehydrogenases.

To our knowledge, the presence of transhydrogenases converting NADPH into NADH has not been reported in yeast. However, a net transhydrogenase activity may derive from the cyclic functioning of pairs of isoenzymes catalysing the same reversible reaction but using the two coenzymes, as has been described by Boles *et al.* (1993) who proposed that growth on glucose of the *S. cerevisiae pgi1* mutant was restored by overproduction of the NAD-glutamate dehydrogenase (coded by *GDH2*); thus inducing NADPH-glutamate dehydrogenase (coded by *GDH1* or *GDH3*) and creating a cyclic transhydrogenase system that transforms NADPH into NADH and regenerates $NADP^+$ for the pentose phosphate pathway, NADH being oxidized by external mitochondrial dehydrogenases. We analyzed the arrays looking for a possible role of mRNA level regulation in the operation of a similar cycle in the *K. lactis rag2* mutant growing in glucose. Fig. 1D shows the relative levels of expression of the glutamate dehydrogenase genes for the *rag2* mutant growing in glucose or fructose. For *GDH2*, signals were very low or zero, *GDH1* and *GDH3* expression seem slightly lower in fructose.

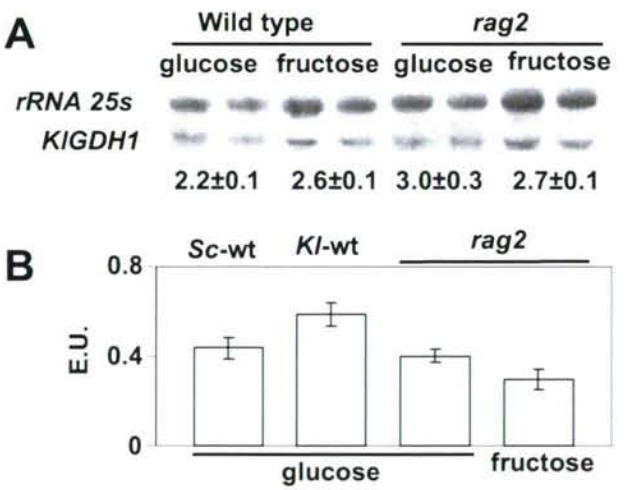

Figure 5: (A) Northern blot analysis of the *KlGDH1* gene expression from the wild type and *rag2* strains of *K. lactis* in glucose and fructose media. n=2. Normalised values of expression are calculated as the quotient of the signals *KlGDH1/rRNA25S* multiplied by 10. Densitometric raw data *KlGDH1/rRNA25S* are as follows; experiment 1, wt G 1111/4716, wt F 1405/5355, *rag2* G 1237/4565, *rag2* F 1252/4854; experiment 2, wt G 926/4294, wt F 1216/4847, *rag2* G 1209/3664, *rag2* F 1204/4256 (B) NADPH-glutamate dehydrogenase activity in the same conditions described in Fig. 5A and in the *S. cerevisiae* wild type strain in glucose media. n=4 for *S. cerevisiae*, n=6 for wild type *K. lactis* and n=5 for the *rag2* mutant. E.U., enzyme units.

We decided to investigate *KlGDH1* mRNA levels and enzyme activity from cells grown in minimal medium (CM but without amino acids, except those required because of auxotrophic mutations) because in complete medium the intensity of the signal in the Northern blot was very low (data not shown). Northern blot with an homologous *KlGDH1* probe (in *K. lactis* only a partial sequence homologous to *GDH1* is available, Valenzuela *et al.*, 1995) shows no increase in the mRNA levels of *KlGDH1* in the *rag2* mutant growing in glucose versus fructose or the wild type strain (Fig. 5A). Enzyme activities show a slight increase (1.3-fold) in the *rag2* mutant growing in glucose versus fructose (Fig. 5B) but that is not sufficient to accommodate the estimated in vivo NADPH turnover derived from the glucose deviation through the oxidative part of the pentose phosphate pathway (calculations not shown). Therefore we cannot conclude that the glutamate dehydrogenase functions as a transhydrogenase in *K. lactis*.

Boles *et al.* (1993) restored growth on glucose of the *S. cerevisiae* mutant in phosphoglucose isomerase (*pgi1*) by adding oxidizing agents like hydrogen peroxide or menadione, which cause oxidative stress to the yeast cells. The oxidative stress response of *S. cerevisiae* includes up-regulation of genes coding for enzymes to keep glutathione and thioredoxin reduced (Koerkamp *et al.*, 2002), which use NADPH as cofactor. *S. cerevisiae* cells obtain the extra NADPH necessary in these conditions by redirecting carbohydrate fluxes to the pentose phosphate pathway at the expense of glycolysis (Godon *et al.*, 1998). We have assayed in this work whether metabolic reactions of defence to oxidative stress, being NADPH-dependent, are induced in the *K. lactis rag2* mutant growing in glucose. This is an attractive hypothesis since the high activity of the respiratory chain in these conditions (González Siso *et al.*, 1996a) could increase intracellular levels of ROS (reactive oxygen species), which would set up the oxidative stress response. It has been estimated that about 2% of total oxygen uptake undergoes incomplete reduction to water during mitochondrial respiration and generates reactive oxygen species (Michiels *et al.*, 2002).

A moderate increase in transcript levels of some genes involved in the defence against oxidative stress is observed when comparing the *rag2* mutant in glucose versus fructose (Fig. 1D). The group of genes whose mRNA levels increase includes: the gene of catalase A (*CTA1*), *ATX1* (copper chaperone), *SKN7* (transcription factor), two genes of the thioredoxin system (*AHP1* and *TRX3*) and a number of genes of the glutaredoxin system (*GRX1*, *TTR1* (*GRX2*), *GRX3*, *GRX4*, *HYR1*). Thus, the glutathione-glutaredoxin system seems to play a role in controlling the redox balance in the *K. lactis rag2* mutant. On the other hand, *OYE2* whose *K. lactis* homologue (*KlYE1*) has been cloned and that codes for a NADPH dehydrogenase of unknown physiological function (Miranda *et al.*, 1995) is not induced in the arrays (Fig. 1D)

although the *S. cerevisiae OYE2* is transcriptionally induced by oxidative stress (Gasch *et al.*, 2000). This was corroborated by Northern blot with a homologous *KlYE1* probe (data not shown). *OYE3*, with no detectable signal in the arrays and whose *K. lactis* homologue has not been cloned hitherto, also codes for a NADPH dehydrogenase that is stimulated in *S. cerevisiae* under oxidative stress (Godon *et al.* 1998).

At present, there is only one report of a *K. lactis* gene involved in the oxidative stress response, the transcriptional factor Yap1p (Billard *et al.*, 1997). We have cloned and characterized the *K. lactis* genes homologous to the *S. cerevisiae GLR1* (glutathione reductase) and *TRR1* (thioredoxin reductase) and regulatory differences with reference to the *S. cerevisiae* homologous genes are found (Tarrio *et al.*, 2004). Data obtained in this work also support the importance of future studies on the NADPH-dependent mechanisms of defence to oxidative stress in *K. lactis* in comparison with the yeast model *S. cerevisiae*.

Acknowledgements

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CHAPTER 4

The nuclear genes encoding the internal (*KlNDI1*) and external (*KlNDE1*) alternative NAD(P)H:ubiquinone oxidoreductases of mitochondria from *Kluyveromyces lactis*

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SUMMARY

Cloning, sequence and functional analysis of the *Kluyveromyces lactis* genes *KlNDI1* and *KlNDE1* are reported. These genes encode for proteins with high homology to the mitochondrial internal (Ndi1p) and external (Nde1p) alternative NADH:ubiquinone oxidoreductases from *Saccharomyces cerevisiae* and complement the respective mutations. Analysis of *KlNDI1* transcriptional regulation showed that expression of this gene is lower in 2% glucose than in 0.5% glucose or non-fermentable carbon sources. β-galactosidase activity values, shown by *lacZ* fusions of *KlNDI1* promoter deletions, suggested that two Adr1p binding sites mediate this carbon source regulation of *KlNDI1*. Expression of the *KlNDE1* gene in *S. cerevisiae* mutant strains and measurement of respiration with isolated mitochondria showed that the protein encoded by *KlNDE1* oxidizes NADPH, this being an important difference with respect to the conventional yeast *Saccharomyces cerevisiae*. Moreover, Northern blot experiments using a phosphoglucose isomerase mutant showed that *KlNDE1* gene transcription increases with glucose metabolism through the pentose phosphate pathway.

INTRODUCTION

Mitochondrial NAD(P)H:ubiquinone oxidoreductases are key enzymes involved in the regulation of intracellular redox balance and energy production, constituting the main entry point for electrons in the mitochondrial respiratory chain. Two types of NAD(P)H:ubiquinone oxidoreductases are found in the mitochondrial inner membrane of eukaryote cells: complex I and the so-called "alternative" or class 2 NAD(P)H:ubiquinone oxidoreductases. In contrast to complex I, alternative NAD(P)H:ubiquinone oxidoreductases are non-proton- pumping, rotenone-insensitive and single polypeptide enzymes of 50-60 kDa molecular mass, whose only prosthetic group is FAD. Alternative enzymes are internal (the catalytic site faces the matrix) or external (the catalytic site faces the intermembrane space) (Kerscher, 2000; Josep-Horne *et al.*, 2001).

In the last few years several alternative NAD(P)H:ubiquinone oxidoreductases from different origins have been characterized, however, their precise metabolic role has not been completely established. In the two extreme situations we found mammals that only have the proton-pumping complex I vs. plants and the fungus *Neurospora crassa* that contain up to four non-proton-pumping alternative enzymes in addition to complex I (Møller, 2002; Duarte *et al.*, 2003). Yeasts are interesting model organisms

since they represent a variety of intermediate situations between mammals and plants. *Yarrowia lipolytica* contains only one external alternative dehydrogenase in addition to complex I (Kerscher *et al.*, 2002), whereas *S. cerevisiae* has replaced complex I by one internal alternative dehydrogenase and also contains two external alternative enzymes (Marres and Vries, 1991; Small and McAlister-Henn, 1998; Luttik *et al.*, 1998). These two species belong to different groups depending on their respiro-fermentative metabolism (Gancedo and Serrano, 1989), *Y. lipolytica* being an obligate aerobe and *S. cerevisiae* a facultative and Crabtree-positive (fermentative) yeast. The genes encoding the alternative dehydrogenases of *Y. lipolytica* and *S. cerevisiae* are the only ones among yeasts that had been characterized up to this work. The study of alternative dehydrogenases of other yeasts will contribute not only to our understanding of the biology of mitochondrial coenzyme reoxidation but also to know the molecular basis of the different patterns of the respiro-fermentative metabolism in yeasts.

Kluyveromyces lactis is a facultative and Crabtree-negative (respiratory) yeast. The higher respiratory capacity of *K. lactis* as compared to *S. cerevisiae* has been attributed to several factors, mainly, to a lower degree of catabolite repression of respiratory genes and higher activity of the pentose phosphate pathway which is coupled to the mitochondrial respiratory chain (González Siso *et al.*, 2000). As with *S. cerevisiae*, the absence of complex I from *K. lactis* mitochondria was evidenced long ago (Josep-Horne *et al.*, 2001) but the alternative dehydrogenases were completely unknown up to this work. We hypothesized the existence of an external NADPH dehydrogenase based on metabolic data obtained from the phosphoglucose isomerase mutant (González Siso *et al.*, 1996a). The more recent demonstration that, differently from *S. cerevisiae*, isolated *K. lactis* mitochondria oxidize NADPH (Overkamp *et al.*, 2002), further supported our hypothesis and definitively established *K. lactis* as an adequate model organism for the study of mitochondrial oxidation of coenzymes.

In this work we isolated and characterized two *K. lactis* genes encoding one internal and other external alternative mitochondrial dehydrogenases, based on their homology with the *S. cerevisiae* genes *NDI1* and *NDE1*, hence termed *KlNDI1* and *KlNDE1*. *KlNDI1* shows induction by non-fermentable carbon sources at the transcriptional level, probably mediated by the factor Adr1p. This induction is not shared by *KlNDE1*. *KlNDE1*, whose protein product uses NADPH as substrate, increases its expression with the activation of the oxidative branch of the pentose phosphate pathway. We propose that the different characteristics of the alternative dehydrogenases of *K. lactis* and *S. cerevisiae* are related to the also different pattern of respiro-fermentative metabolism in these yeasts. The presence of an alternative

internal dehydrogenase instead of complex I characterizes both facultative yeasts. However, the ability of the *K. lactis* external dehydrogenase to use NADPH supports the higher activity of the pentose phosphate pathway, and the absence of down-regulation at high glucose concentrations supports the Crabtree-negative phenotype.

MATERIALS AND METHODS

Cloning of the gene and analysis of the sequence

A *K. lactis* genomic library in the shuttle plasmid KEp6 (Wésolowski-Louvel *et al.*, 1998) was used to search for clones containing genes homologous to the *S. cerevisiae NDI1* and *NDE1* by the PCR-based strategy described in Díaz Prado *et al.* (2004). About 40,000 bacterial colonies were screened using oligonucleotide pairs TGAAGAAAGCCAATTTCAACC – TTGAAG CTTTGCCAAAGC and TGGATCTGACAGGTTCGACAA - TTCGCTCGCGGTGCTAGATT that amplified partial sequences of 704 and 415 bp, respectively, available at GenBank databases (accession numbers AL427909 and AL424975, respectively).

DNA sequencing was performed by primer walking on both strands and using an automated system (Voss *et al.*, 1993).

Computer searches for binding sites for transcriptional factors were performed with the bio-informatics facilities of TFSEARCH (http://www.cbrc.jp/htbin/nph-tfsearch) and TRANSFAC (http://transfac.gbf.de/cgi-bin/matSearch/matsearch2.pl). The homology of the protein sequences was analyzed using BLASTA from NCBI Tools (http://ncbi.nlm.nih.gov/). Multiple alignments were carried out by CLUSTAL W from EBI (http://www.ebi.ac.uk/clustalw/). Prediction of the secondary structure of the proteins was carried out by the program 123D+ (http://123d.ncifcrf.gov/123D+). Prediction of mitochondrial targeting sequences was carried out by the program MitoProt II 1.0a4, available at ExPASy (http://us.expasy.org).

Strains, media and culture conditions

The following *K. lactis* strains were used: the wild type NRRL-Y1140 (*MATa*, ATCC8585, CBS2359), the *rag2* (phosphoglucose isomerase) mutant PM5-2D (*MATa uraA1-1 metA1-1 argA1-1 trpA1-1 rag2-1*), the β-galactosidase deficient mutant MW190-9B (*MATa lac4-8 uraA Rag*$^+$) and the *hap3* mutant WMH7302-D1 (*MATα leu2 uraA trp1 metA1 ade2 his2-2 hap3::ScLEU2*). PM5-2D and MW190-9B were kindly provided by Dr. Wésolowski-Louvel (France). WMH7302-D1 was kindly provided by Dr. L. A. Grivell (The Netherlands).

The following *S. cerevisiae* strains, supplied by Euroscarf (http://www.uni-frankfurt.de/fb15/mikro/euroscarf/col_index.html), were used: BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ*), BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ*), Y06521 (BY4741 *YML120c::kanMX4*), Y16521 (BY4742 *YML120c::kanMX4*), Y00726 (BY4741 *YMR145c::kanMX4*). The ORFs YML120c and YMR145c correspond to the genes *NDI1* and *NDE1*, respectively. The *S. cerevisiae* strains ENY.WA-1B (*MATa ura3-52 leu2-3,112 trp1-289 MAL2-8^c MAL3 SUC3*) and the phosphoglucose isomerase mutant EBY22 (ENY.WA-1B *pgi1Δ::TRP1*) were kindly provided by Dr. E. Boles (Germany).

Growth and handling of yeasts were carried out according to standard procedures (Kaiser *et al.*, 1994). The yeast cells were cultivated, unless otherwise stated, in Erlenmeyer flasks at 30°C and 250 rpm in the synthetic complete medium CM (Zitomer and Hall, 1976), the dropout medium CM-ura (without uracil) or YP medium (2% bactopeptone, 1% yeast extract) containing one of the following carbon sources: 0.5% glucose, 2% glucose, 2% lactate, 2% glycerol, 2% ethanol, 2% fructose (supplemented with 0.05% glucose for PM5-2D). Solid growth media also contained 1.5% agar.

To generate oxidative stress conditions, cells were grown in CM medium up to OD_{600}=0.6 and then switched to CM+H_2O_2 for 15 minutes, hydrogen peroxide added to a final concentration of 0.4 mM (Tarrío *et al.*, 2004).

The effect of oxygen supply upon transcription was measured as follows: the cells of the wild type *K. lactis* strain were grown in a 2-L vessel fermentor (Biostat MD; Braun-Biotech, Germany) in YP-2% glucose supplemented with 0.002% ergosterol. The working volume of the culture was 1.5 L and temperature was maintained at 30°C. The air flow was 180 L/h (2 v.v.m.) sparged through the culture, which was agitated at a speed of 300 rpm. Nitrogen (99.95% pure;

Carburos Metálicos, Spain) was bubbled at a constant pressure of 0.15 bars. Dissolved oxygen was measured with a polarographic electrode. The oxygen electrode was calibrated prior to inoculation of the cultures by equilibration with air (full scale, pO2 = 100%) and nitrogen (zero, pO2 = 0%). Cells were grown under nitrogen flow up to OD_{600}=0.5 when nitrogen was replaced with air. Several samples were taken thereafter for RNA extraction at different time intervals up to 2 hours. Cells were harvested by a rapid sampling procedure and quick-chilled in an ice-bath, immediately pelleted by centrifugation, washed, flash-frozen in liquid nitrogen and stored at -80°C.

RNA extraction and Northern blot analysis

These procedures were performed as described earlier (González-Domínguez *et al.*, 2000). The *KlNDI1* and *KlNDE1* probes were obtained by PCR amplification of genomic DNA from the *K. lactis* strain NRRL-Y1140 with specific oligonucleotides, and comprised, respectively, 90% of the coding sequence from nucleotide +106 to +1513 (*KlNDI1*) and 50% of the coding sequence from nucleotide +315 to +1130 (*KlNDE1*). Northern blot hybridization was quantified using a PhosphorImager Scanning Instrument 425 and the ImageQuant program (Molecular Dynamics). Data were normalized for RNA loading against the signal obtained from the control probe rRNA 25S. Several independent RNA extractions/hybridizations were performed for each strain/condition and results averaged.

RNA extraction and Real-Time Reverse-Transcription PCR

The following kits and equipment were used, following the instructions and the conditions recommended by the suppliers: "Perfect RNA™ Eukaryotic Mini" (Eppendorf) for RNA extraction, "iScript cDNA Synthesis" (Bio-Rad) and "GeneAmp PCR System 2700" (Applied Biosystems) for the reverse transcription PCR, "iQ SYBR Green Supermix" and "iCycler iQ" (Bio-Rad) for real-time PCR detection. The iCycler software (v 3.0a) was used for data analysis. Relative levels of expression were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). As internal controls the *K. lactis ACT1* (actin) and *GAP1* (glyceraldehyde-3-phosphate dehydrogenase) genes were used, and specific regions in the ORFs of 299 and 298 bp were amplified with the respective pairs of primers (TGGAAAGTGGTCAAAGAGGC – CGAAAGATTCAGAGCTCCAG and GGGTGGATCTTTTTCATTTTG – TTAACGGCAACAAC TTCTAG). The *KlNDI1* gene (a region of 235 bp in the ORF) was amplified with primers GTTTGGCAAAGCTTCAACCA and CCCTCACTCTTTGCAAATCA. Agarose gel electrophoresis of the PCR products revealed a single band corresponding to the single-amplified products as predicted by the melting curve analysis of the PCR. The amplification efficiency was 98-99% for both the target and internal control genes.

Isolation of mitochondria

The method described by Herrmann *et al.* (1994) was used. Cells were aerobically grown in CM or CM-ura (transformants) with 2% lactate as carbon source.

Oxygen uptake studies with mitochondrial preparations

Substrate-dependent oxygen consumption rates of freshly isolated mitochondria were determined at 30°C using a Clark-type oxygen electrode (Hansatech) as described by Luttik *et al.* (1998). Respiratory substrates were 0.2 mM NADH and 1 mM NADPH (Overkamp *et al.*, 2002). Measurements were made in the absence or presence of 0.25 mM ADP, and all respiration was found to be sensitive to antimycin A.

Protein determination

Protein concentration was measured by the method of Bradford (1976) using bovine serum albumin as a standard.

Promoter-*lacZ* fusions and β-galactosidase activity

Eight unidirectional deletions were constructed by PCR amplification of different size *KlNDI1* promoter fragments from nucleotides -866 to +3 and cloning the amplified products in the *Bam*HI-*Hind*III sites of pXW2 (Chen *et al.*, 1992) fused in frame to the 8th codon of the *lacZ* reporter gene. The resultant constructions were named PIF1 (-866), PIF2 (-805), PIF3 (-700), PIF4 (-600), PIF5 (-490), PIF6 (-408), PIF7 (-300) and PIF8 (-207); their correctness was verified by sequencing. A second set of constructions resulted from fusing selected promoter regions 5′ to the shortest promoter region of PIF8, and these were named PIF1-8 (-847 to -806),

PIFA (-800 to -779) and PIFG (-822 to -801 and -724 to -704). Again, their correctness was verified by sequencing. All of these *lacZ* fusions and the empty vector were used to transform the *K.lactis* β-galactosidase deficient strain MW190-9B.

For qualitative determination of β-galactosidase activity, individual transformed colonies were grown on plates with the chromogenic substrate X-gal (Rose *et al.*, 1990) and blue colour appearance was analyzed.

For quantification of β-galactosidase activity, three to five transformed colonies were picked randomly and grown in CM-ura up to OD_{600} of approximately 0.6. β-galactosidase assays were performed with permeabilized cells and Enzyme Units were calculated as described in Adams *et al.* (1997). Values represent the average of at least three independent determinations of each transformed colony.

Statistical analysis

Data are expressed as mean ± standard deviation (S.D.). The statistical significance of differences found between means was evaluated at the 95% confidence level by the ANOVA and Multiple Comparisons Tests performed by the programs StatGraphics Plus or GraphPad Instat.

Other procedures

Standard procedures for manipulation of nucleic acids were essentially those of Sambrook *et al.* (1989). *Escherichia coli* DH-10B was used for plasmid amplification by electroporation. Yeast transformation was performed using the lithium acetate procedure (Gietz and Woods, 1994) or by electroporation. Electroporations were carried out using a BIO-RAD Gene Pulser II and following the instructions of the supplier.

RESULTS

Cloning of the *KlNDI1* and *KlNDE1* genes and analysis of the sequences

The DCbyPCR procedure (Díaz Prado *et al.*, 2004) was used, as described in Materials and methods, to isolate two clones from a *K. lactis* genomic library containing full-length genes with significant similarity to the *S. cerevisiae* ORFs YML120c (*NDI1*) and YMR145c (*NDE1*) that were therefore named *KlNDI1* and *KlNDE1*. The sequences were submitted to EMBL/GenBank/DDBJ Databases (accession numbers AJ496545 and AJ496546, respectively). The nucleotide sequence of *KlNDI1* contains the CDS (1560 bp), an upstream region of 1274 bp and a downstream region of 1114 bp. The nucleotide sequence of *KlNDE1* contains the CDS (1644 bp), an upstream region of 1536 bp and a downstream region of 656 bp.

Based on computer analysis, the sequence information indicates that *KlNDI1* and *KlNDE1* code for mitochondrial proteins of 519 and 547 amino acids, respectively, with 41.6% identity between them. Similarly, 41.5% identity exist between *S. cerevisiae* Ndi1p and Nde1p (Luttik *et al.*, 1998). The putative proteins Klndi1p and Klnde1p show 72% and 67% identity, respectively, with the *S. cerevisiae* homologues Ndi1p and Nde1p. Klnde1p shows lower identity with the other alternative external dehydrogenases present in the ClustalW of Fig. 1, i.e., the enzymes from *Neurospora crassa* (34% with Nde1p, 50% with Nde2p) or *Solanum tuberosum* (44% with Ndb1p), which have been reported to oxidize NADPH (Melo *et al.*, 2001; Carneiro *et al.*, 2004;

Michalecka *et al.*, 2004). *N. crassa* Nde1p and *S. tuberosum* Ndb1p show a calcium binding motif (Melo *et al.*, 1999; Rasmusson *et al.*, 1999) that is absent from Klnde1p.

Fig. 1 shows that both *K. lactis* proteins contain two conserved glycine-rich (GXGXXG) motifs in predicted βαβ domains, hence meeting the criteria for binding of the substrate NAD(P)H or the non-covalently attached FAD cofactor (Lesk, 2001). One of these lies close to the N-terminus (residues 66-71 of Klndi1p and 107-112 of Klnde1p) and shows the last of the three highly conserved glycine residues replaced by an alanine (Klndi1p) or serine (Klnde1p). This replacement is also observed in the proteins from *S. cerevisiae* and *N. crassa*, but not in the protein from *S. tuberosum*. The other motif is found 175 (Klndi1p) or 157 (Klnde1p) amino acids further downstream. Melo *et al.* (1999, 2001) speculated that the first glycine-rich motif of *N. crassa* Nde1p binds NADPH because a conserved negative charge at the end of the second β sheet is substituted by an Asn (N167), avoiding the unfavourable interaction between the negatively charged residue and the negatively charged 2´-phosphate of NADPH. However, the other alternative dehydrogenases present in the ClustalW of Fig. 1 have a conserved Asp in this position, even the *S. tuberosum* Ndb1p, NADPH-specific (Michalecka *et al.*, 2004). Therefore, coenzyme specificity of Klnde1p cannot be predicted from the sequence.

As predicted by the program MitoProt II (Claros and Vicens, 1996), Klndi1p includes an N-terminal mitochondrial-targeting sequence of 25 residues (probability of 0.92) and Klnde1p includes an N-terminal mitochondrial-targeting sequence of 56 residues (probability of 0.99). The prediction that the proteins coded by *KlNDI1* and *KlNDE1* are, respectively, internal and external NAD(P)H:ubiquinone oxidoreductases, is supported by the characteristics of these N-terminal sequences. The two external NADH:ubiquinone oxidoreductases of mitochondria from *S. cerevisiae,* Nde1p and Nde2p, in relation to Ndi1p, have been reported to show N-terminal extensions of 30-45 amino acids that may be involved in targeting of the proteins to the appropriate subcellular locations (Luttik *et al.*, 1998). The same occurs with the homologous *K. lactis* proteins, KlNdi1p vs. KlNde1p, as shown in Fig. 1. The predicted functions of Klndi1p and Klnde1p as well as the ability of Klnde1p to oxidize NADPH have been experimentally demonstrated as described in the following sections.

Figure 1: (A) ClustalW alignment of the sequences of the mitochondrial internal (NDI1) and external (NDE1, NDE2) NAD(P)H:ubiquinone oxidoreductases from *Saccharomyces cerevisiae* (Sc) and *Kluyveromyces lactis* (Kl) identified hitherto, and also of the external enzymes using NADPH from *Neurospora crassa* (Nc) and *Solanum tuberosum* (St). Glycine-rich motifs for coenzyme binding are in bold and shadowed. In the *K. lactis* proteins, the putative mitochondrial targeting sequences are underlined.

The cloned *KlNDI1* gene can complement the *ndi1* mutation of *S. cerevisiae in vivo*

Saccharomyces cerevisiae mutants with a deletion in the *NDI1* coding sequence are unable to grow in CM with 2% lactate as sole carbon source (Marres and Vries, 1991). We used this phenotype, which is not shared by the *NDE1* or *NDE2* mutants (Small and McAlister-Henn, 1998), to prove the function of Klndi1p by heterologous complementation. For this purpose, the *KlNDI1* coding sequence flanked by 400 bp upstream and 532 bp downstream was cloned into the *Eco*RI site of the vectors YEplac195 and YCplac33 (Gietz and Sugino, 1988). The resultant plasmids and the empty vectors were then introduced into the *S. cerevisiae* strains Y06521 and Y16521 which are devoid of Ndi1p.

Ura+ transformants were selected on CM-ura with glucose as carbon source. They were then tested for their ability to grow on the same medium but with lactate as carbon source. All the transformants containing the *KlNDI1* gene, either in the high or

low copy number vectors, were able to grow on lactate, while the transformants containing the empty vectors were not. The same results were obtained with the original clone in KEp6. When transformed cells were grown under non-selective conditions to allow plasmid loss, both of the phenotypes (+ura, +lactate) were unstable, as expected for a plasmid-based phenotype, i.e., the colonies void of plasmid, unable to grow in CM-ura with glucose, were also unable to grow in CM with lactate. This experiment (data not shown) demonstrated that the *KlNDI1* gene can complement the *ndi1* mutation of *S. cerevisiae* in vivo, and also that the 400 bp *KlNDI1* promoter region is functional in *S. cerevisiae*. Therefore, we conclude that *KlNDI1* indeed codes for a mitochondrial internal NADH:ubiquinone oxidoreductase.

Transcription of *KlNDI1*, but not of *KlNDE1*, is regulated by the carbon source

Analysis of transcription by Northern blot or quantitative RT-PCR (Fig. 2) showed that the levels of *KlNDE1* mRNA in *K. lactis* cells grown in media with high (2%) glucose were similar to those obtained in low (0.5%) glucose or in the non-fermentable carbon source lactate. In contrast, *KlNDI1* mRNA levels were significantly lower when *K. lactis* cells were grown in the media with high glucose than with low glucose or lactate.

The computer searches for putative binding sites of transcriptional factors in the *KlNDI1* promoter showed consensus sequences related to the above described carbon source regulation (Gancedo, 1998): Adr1p, Hap2/3/4/5p and Gcr1p. Therefore, we studied if such consensuses were functional in the transcriptional regulation of *KlNDI1*.

With this objective in mind, first we measured the β-galactosidase activity shown by the β-galactosidase defective *K. lactis* cells transformed with the construction PIF1 (the fusion of the 866 bp *KlNDI1* upstream region to the reporter gene *lacZ* in the plasmid pXW2) as described in Materials and methods. The colours of the colonies on X-gal plates with different carbon sources (not shown), white on 2% glucose and blue on 0.5% glucose and on non-fermentable carbon sources (lactate, glycerol, ethanol), supported the result exposed above of lower expression on 2% glucose, as did the quantification of the β-galactosidase activity of the transformants grown in liquid medium with the same carbon sources (Fig. 3A).

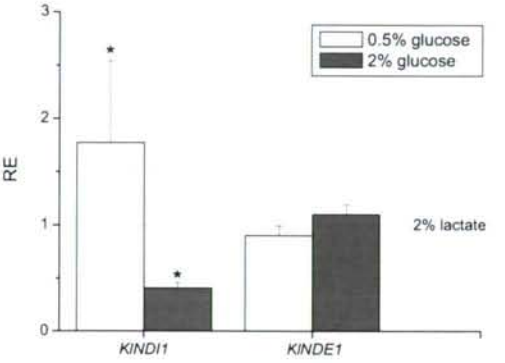

Figure 2: Effect of the carbon source on *KlNDI1* and *KlNDE1* transcription: Relative expression (RE) levels of the *KlNDI1* and *KlNDE1* genes from the *K. lactis* wild type strain cultured in media with 0.5% glucose, 2% glucose and 2% lactate as carbon sources. mRNA levels were measured by Northern blot (*KlNDE1*) or quantitative RT-PCR (*KlNDI1*), as described in Materials and methods. Northern blot results are the mean ± S.D. of 2 measurements. Quantitative RT-PCR results are the mean ± S.D. of 16 measurements. Data were normalized vs. the values obtained in lactate which were considered equal to 1. * indicates significant differences with the control (lactate). *KlNDI1* mRNA levels were also measured by Northern blot, the result was a 2.3-fold increase of *KlNDI1* transcription in 0.5% glucose vs. 2% glucose.

Then, and once verified that the 866 bp upstream regulatory region of the *KlNDI1* gene was able to exert the carbon source dependent transcriptional regulation of the reporter gene, we measured the β-galactosidase activity shown by the *K. lactis* cells transformed with the constructions PIF2 to PIF8 carrying the fusions to *lacZ* of the *KlNDI1* promoter unidirectional deletions, as described in Materials and methods. The deletions were delimited as a function of the consensus sequences for putative binding of transcription factors found in the *KlNDI1* promoter region. Fig. 3A shows that β-galactosidase activity values in cells transformed with PIF2 (805 bp promoter) confirmed the decrease in promoter activity exerted by 2% glucose, although less pronounced than with PIF1. The shortening of the promoter up to nucleotide -700 (PIF3) provoked the disappearance of this effect, and thus cells transformed with the constructions PIF3, PIF4 (600 bp promoter) and PIF5 (490 bp promoter) showed similar activity in 0.5% and 2% glucose, this activity being significantly higher than in 2% lactate. The promoter region that had proved to be functional in the experiments of heterologous complementation is represented by PIF6 (408 bp), that showed lower activity than with PIF1 but significantly higher than the control (cells transformed with the empty pXW2 vector) both in glucose and lactate. The shortest promoter region assayed (PIF8, 207 bp) showed similar activity in glucose and lactate, this activity being at the same level as with PIF7 (300 bp promoter) and PIF6. Therefore, we

considered that PIF8 contained the shortest promoter that would be useful for the following experiments.

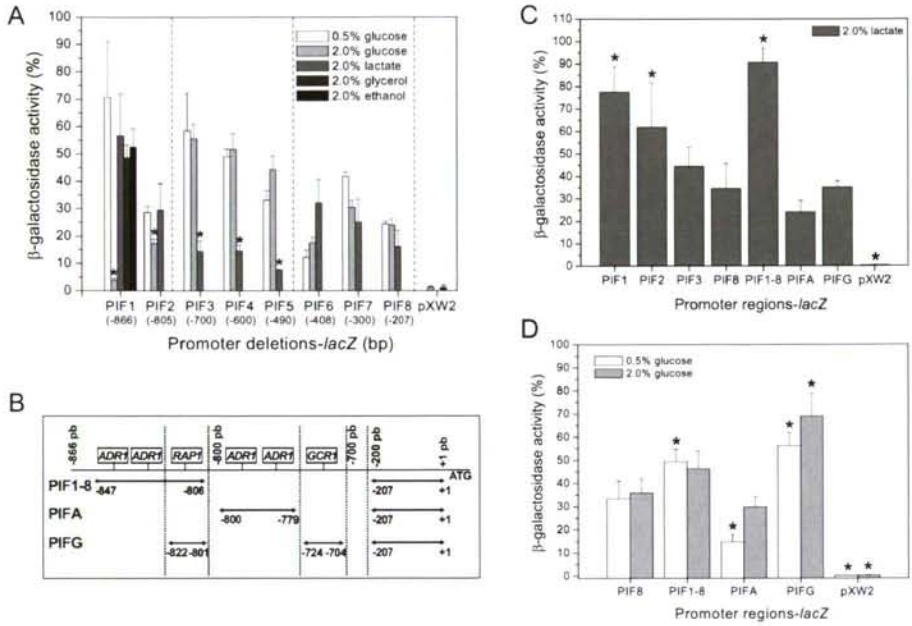

Figure 3: Assay of the functionality of putative binding sites for transcription factors involved in carbon source regulation from the *KlNDI1* promoter region. A) β-galactosidase activities shown by the transformants of the MW190-9B strain with the fusions to *lacZ* in pXW2 of unidirectional deletions of the *KlNDI1* 866 bp promoter region, cultured in media with 0.5% glucose, 2% glucose and 2% lactate as carbon sources. Cells transformed with PIF1 were also cultured in media with 2% glycerol and 2% ethanol as carbon sources. Data are the mean ± S.D. of three to five independent transformants assayed in triplicate and were normalized considering that the maximum value was 100%. * indicates significant differences with the rest of conditions of the same construction. B) A scheme of the fusions of the selected sequences to the shortest promoter (-207 bp) in pXW2, performed as described in Materials and methods. C) β-galactosidase activities shown by the transformants of the MW190-9B strain with the fusions to *lacZ* in pXW2 described in B, cultured in media with 2% lactate as carbon source. Data are the mean ± S.D. of three independent transformants assayed in triplicate and were normalized considering that the maximum value was 100%; * indicates significant differences with PIF8. D) β-galactosidase activities shown by the transformants of the MW190-9B strain with the fusions to *lacZ* in pXW2 described in B, cultured in media with 0.5% glucose and 2% glucose as carbon sources. Data are the mean ± S.D. of three independent transformants assayed in duplicate. * indicates significant differences with PIF8.

From the comparison of the experimental results obtained with the unidirectional deletions and the position of the consensus sequences for putative binding of transcription factors found by in silico analysis in the *KlNDI1* promoter region, we observed that the region from -866 to -700, which seemed to be responsible for the higher activity in lactate and 0.5% glucose vs. 2% glucose, did not contain putative binding sites for repressors. However it contained four putative binding sites

for Adr1p, one for Hap2/3/4/5p, one for Rap1p and one for Gcr1p. To test the functionality of these binding sites, we fused the corresponding sequences to the shortest promoter in PIF8 (see Fig. 3B) and measured the β-galactosidase activity of the reporter. We also measured, by quantitative RT-PCR, the *KlNDI1* mRNA levels in a *K. lactis* strain lacking Hap3p of the complex Hap2/3/4/5p vs. the wild type strain.

β-galactosidase activity in lactate and glucose measured from the cells transformed with the fusions to the shortest promoter (PIF8) is shown in Fig. 3C and D. The fusion of the sequence from -866 to -806 (PIF1-8), containing two putative Adr1p binding sites and the Rap1p binding site, restored the same levels of activity in lactate shown by PIF1. However, the fusion of the sequence from -800 to -779 (PIFA), containing two other putative Adr1p binding sites, did not increase the activity in lactate when compared to PIF8. The fusion of the sequences of the putative Gcr1p and Rap1p binding sites (PIFG) caused a significant increase in activity in reference to PIF8 in glucose (0.5 and 2%) but not in lactate. The consensuses for Gcr1p and Rap1p are further apart in the *KlNDI1* promoter (90 bp) than in PIFG (13 bp) since in the construction we removed the sequence containing the binding sites for Adr1p and Hap2/3/4/5p. Hence, we cannot strictly ensure that this activating effect in glucose is operative in the native *KlNDI1* promoter. Moreover, quantitative RT-PCR analysis showed that *KlNDI1* mRNA levels in the *K. lactis Δhap3* mutant were 1.4-fold lower than in the wild type strain grown in media with lactate as carbon source (the relative expression in the *Δhap3* vs. the wild type strain was 0.70 ± 0.17, mean ± SD of 18 samples). These results suggest that Adr1p and, probably, Hap2/3/4/5p, Gcr1p and Rap1p are involved in carbon source regulation of *KlNDI1*.

KlNDI1 transcription is not regulated by oxygen

We also assayed the influence of oxygen availability on *KlNDI1* mRNA levels. Total RNA was extracted from cells cultured under conditions of different oxygen/nitrogen ratios, obtained as described in Materials and methods. *KlNDI1* mRNA levels, measured by Northern blotting, were neither significantly different in cultures grown under nitrogen flow vs. fully-aerobic conditions (not shown) nor during the shift hypoxia-aerobiosis (Fig. 4). Although in Fig. 4 only the results of 30 minutes are shown, samples were taken up to 2 hours, reaching a similar conclusion.

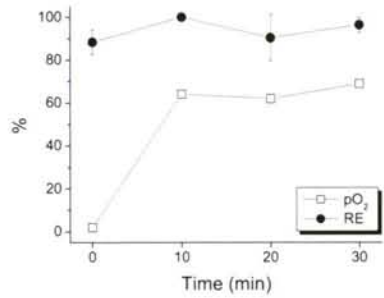

Figure 4: Effect of dissolved oxygen levels on *KlNDI1* transcription during the transition from hypoxic to aerobic conditions, measured by Northern blotting. Results are the mean ± S.D. of two samples. For conditions see Materials and methods.

The protein encoded by *KlNDE1* uses NADPH as substrate

Our interest in the isolation and study of the *KlNDE1* gene mainly focused on testing the hypothesis that, differently from *S. cerevisiae*, a *K. lactis* mitochondrial external alternative dehydrogenase could oxidize cytosolic NADPH, thus allowing the higher activity of the pentose phosphate pathway and the growth in glucose of the phosphoglucose isomerase mutant of *K. lactis* (González Siso *et al.*, 1996a).

For this purpose, the *KlNDE1* coding sequence flanked by 1088 bp upstream and 656 bp downstream was cloned into the *Eco*RI-*Sal*I sites of the vector YEplac195 (Gietz and Sugino, 1998). The plasmid obtained and empty vector were then introduced into the *S. cerevisiae* strain EBY22 which is devoid of phosphoglucose isomerase and unable to grow in glucose (Boles *et al.*, 1993). The transformants with the plasmid carrying the *KlNDE1* gene were able to grow on CM-ura with glucose (0.1%, 0.5% and 2%), although the growth rate was lower than that of the *S. cerevisiae* wild type strain, whereas those with the empty plasmid were not (not shown). Since it has been reported that the inability to grow in glucose of the *S. cerevisiae* phosphoglucose isomerase mutant is due to a depletion of $NADP^+$ that inhibits the pentose phosphate pathway (Boles *et al.*, 1993), the result exposed above suggests that the protein encoded by *KlNDE1* is able to oxidize the cytosolic NADPH produced in the pentose phosphate pathway. To obtain more evidence supporting this conclusion, we also transformed, with the same construction, the *S. cerevisiae nde1* mutant strain Y00726 and measured the rates of oxygen consumption by isolated mitochondria from the transformants, with NADH and NADPH as substrates. Table 1 clearly shows that the mitochondria isolated from the *S. cerevisiae nde1* mutant expressing the *K. lactis NDE1* gene oxidize externally added NADPH, although the rate is lower than in the wild type *K. lactis* strain (Overkamp *et al.*, 2002), whereas

mitochondria isolated from the *S. cerevisiae* wild type strain do not, as previously reported (Small and McAlister-Henn, 1998; Luttik *et al.*, 1998). Moreover, the rate of NADH-dependent respiration increases in the transformants vs. the *nde1* mutant up to the level shown by a *K. lactis* wild type strain (Overkamp *et al.*, 2002). Therefore, these results indicate that *KlNDE1* complements the nde1 mutation in *S. cerevisiae* and that Klnde1p oxidizes NADPH and, probably, also NADH. The increase in NADH-dependent respiration may also be caused by a pleiotropic effect via a changed Scnde2p expression.

The observation that the rate of NADPH oxidation by the mitochondria isolated from the *S. cerevisiae nde1* mutant expressing the *K. lactis NDE1* gene is lower than that obtained with mitochondria of the wild type *K. lactis* strain may be due to two reasons: The first is the activation exerted on *KlNDE1* by the NADPH produced in the pentose phosphate pathway (a detailed explanation follows under the next heading); since the pentose phosphate pathway is less active in *S. cerevisiae* than in *K. lactis* (Jacoby *et al.*, 1993), also the levels of NADPH and the expression of *KlNDE1* may be concomitantly lower in *S. cerevisiae*. The second reason is the possible existence in *K. lactis* mitochondria of an additional external alternative dehydrogenase using NADPH. In fact, in the Genolevures database (http://cbi.labri.u-bordeaux.fr/Genolevures/) we found a putative protein (KLLA0A08316g) that meets the required characteristics, although showing only 39% identity to KlNde1p, and its characterization is now in progress.

Table I: Substrate-dependent rates of oxygen consumption by mitochondria from wild type *S. cerevisiae* BY4741 (WT), the *nde1* mutant Y00726 (*nde1*), and two independent transformants with the episomic plasmid carrying the *KlNDE1* gene (DHE1 and DHE10).

STRAIN		NADH	NADPH
WT	Oxygen uptake rate	0.46 ± 0.05	N.D.
	RC	2.01 ± 0.07	N.D.
nde1	Oxygen uptake rate	0.09 ± 0.00	N.D.
	RC	1.32 ± 0.22	N.D.
DHE1	Oxygen uptake rate	0.12 ± 0.03	0.04 ± 0.01
	RC	1.62 ± 0.2	1.37 ± 0.16
DHE10	Oxygen uptake rate	0.13 ± 0.01	0.03 ± 0.00
	RC	1.62 ± 0.02	1.23 ± 0.05

Cells were grown in aerobic lactate cultures as described in Herrmann *et al.* (1994). The oxygen uptake rates given (μmol O_2/min*mg protein) were measured in the presence of 0.25 mM ADP, respiratory control (RC) values represent the ratio of respiration rates in the presence and absence of ADP. The assays were performed at pH = 7.0. Experimental results are the mean ± S.D. of at least two measurements, and in the case of the transformants, two independent mitochondrial isolations for each. N.D. = not detected.

Transcription of *KlNDE1* increases with glucose metabolism through the pentose phosphate pathway

The recently published observation that the NADPH-oxidizing activity of mitochondria isolated from a *K. lactis* phosphoglucose isomerase mutant was 2.5-fold higher than that of a wild type mitochondria (Overkamp *et al.*, 2002) suggests the existence of a relation between the levels of expression of the external *K. lactis* NAD(P)H dehydrogenase and the activity of the pentose phosphate pathway which is the main source of cytosolic NADPH. To study if this occurred at a transcriptional level with the *KlNDE1* gene cloned in this work, we performed a Northern blotting analysis. RNA was extracted from cells of the *K. lactis* wild type and the phosphoglucose isomerase mutant strains grown in CM media with glucose or fructose as carbon sources. The phosphoglucose isomerase mutant diverts all the glucose through the oxidative reactions of the pentose phosphate pathway, to bypass the blocked glycolytic step, with the concomitant increase in NADPH production.

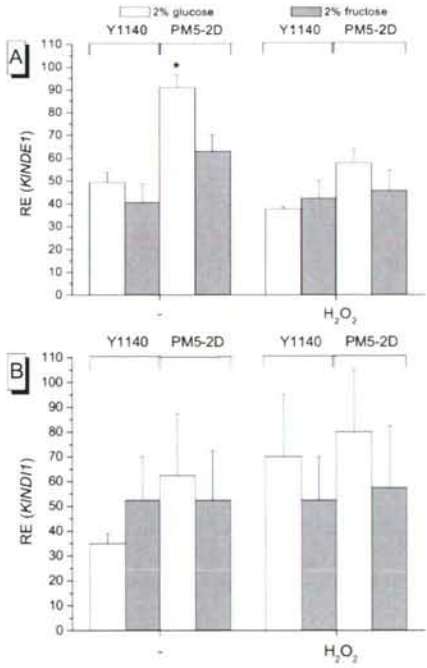

Figure 5: Relative Expression (R.E.) obtained from Northern blotting analysis of the mRNA levels of *KlNDE1* (A) and *KlNDI1* (B) from the wild type (Y1140) and phosphoglucose isomerase mutant (PM5-2D) *K. lactis* strains grown in media with glucose and fructose as carbon sources, before and after addition of 0.4 mM hydrogen peroxide for 15 minutes. Results in A are the mean ± S.D. of four samples (two independent extractions assayed in duplicate) and results in B are the mean ± S.D. of three samples. After loading normalization, the highest intensity in each blot was given a value of 100% and used as a reference for the other signals. * indicates significant differences with the rest of conditions.

The results shown in Fig. 5A suggest that the transcription of the *KlNDE1* gene is regulated by the ratio NADPH/$NADP^+$, which is dependent on the activity of the pentose phosphate pathway, since mRNA levels are significantly higher in the phosphoglucose isomerase mutant strain grown in glucose vs. fructose and vs. the wild type strain. Moreover, Fig. 5A also shows that the induction of the *KlNDE1* gene in the phosphoglucose isomerase mutant strain grown in glucose disappears when 0.4 mM hydrogen peroxide is added to the medium, i.e., when NADPH-consuming mechanisms of defence to oxidative stress, specifically thioredoxin reductase, are induced (Tarrío *et al.*, 2004). To determine if this behaviour of the *KlNDE1* gene was related to the higher activity of the respiratory chain shown by the *K. lactis* phosphoglucose isomerase mutant grown in glucose (González Siso *et al.*, 1996a), we performed another Northern blot analysis under similar conditions but using the *KlNDI1* gene as a probe. There was no variation in *KlNDI1* mRNA levels between the two strains growing in glucose or fructose, with or without hydrogen peroxide (Fig. 5B). Therefore, the increase in the activity of the respiratory chain that has been reported when *K. lactis* uses the pentose phosphate pathway, i.e., when the phosphoglucose isomerase mutant grows in glucose, is correlated with the transcript levels of the NADPH-oxidizing activity of the external dehydrogenase that passes the electrons to the ubiquinone. In fact, such increase was observed in oxygen consumption and *KlCYC1* transcription (González Siso *et al.*, 1996a) and in *KlNDE1* but not in *KlNDI1* transcription (this work).

We conclude that there is a transcriptional induction of *KlNDE1* under conditions of high activity of the oxidative branch of the pentose phosphate pathway and therefore high $NADP^+$/NADPH turnover.

DISCUSSION

Alternative mitochondrial NAD(P)H dehydrogenases are key enzymes of cellular metabolism, however, their precise physiological role is still far from being well understood. These enzymes might constitute a wasteful system acting to prevent the overreduction of the electron transport components and the production of reactive oxygen species (Melo *et al.*, 2001). The simultaneous presence of both complex I and internal alternative NAD(P)H:ubiquinone oxidoreductase has been reported for plants and the filamentous fungus *N. crassa* (Kerscher, 2000; Møller, 2002; Videira and Duarte, 2002). In this case, the internal alternative enzyme may compete with complex I for intra-mitochondrial NADH and ubiquinone. In other organisms, only one of these two systems for respiratory chain oxidation of intra-mitochondrial NAD(P)H is present. External enzymes are one out of several existing systems for feeding electrons from

cytosolic NADH into the respiratory chain (Bakker *et al.*, 2001); some of these also use NADPH (Melo *et al.*, 2001; Carneiro *et al.*, 2004). These enzymes might act as an overflow mechanism for the oxidation of cytosolic NAD(P)H under conditions where the level of these coenzymes is higher than that used in assimilatory reactions (Melo *et al.*, 1999).

In this work, we describe the cloning and characterization of two genes from the yeast *Kluyveromyces lactis* termed *KlNDI1* and *KlNDE1*, homologous to the *S. cerevisiae* genes coding for the internal (*YML120c/NDI1*) and external (*YMR145c/NDE1*) alternative mitochondrial dehydrogenases. *K. lactis* is a yeast that is closely related to *S. cerevisiae* but shows a different respiro-fermentative metabolism (reviewed in González Siso *et al.*, 2000), crucial differences are related to the Crabtree phenotype and the activity of the pentose phosphate pathway.

Both *K. lactis* and *S. cerevisiae* are facultative aerobes and lack complex I (Josep-Horne *et al.*, 2001), so it seems that this type of organisms prefers the more simple alternative internal dehydrogenases to complex I present in strictly aerobic yeasts like *Y. lipolytica* (Kerscher *et al.*, 2002). This may be because the transitions from a fermentative to a respiratory metabolism, and vice versa, are easier to perform with a single polypeptide enzyme doing the function of complex I which is composed of a high number of subunits, even at the expense of a lower P/O ratio.

The results presented in this work point out that *KlNDI1* expression is regulated at the level of transcription by the carbon source, expression being higher when the cells grow in low glucose concentration and in non-fermentable carbon sources than in high glucose concentration. In a similar way, the *S. cerevisiae* Ndi1p is more abundant in cells grown in non-fermentable carbon sources than in glucose (de Vries and Grivel, 1988) and transcription of the gene increases after the diauxic shift (de Risi *et al.*, 1997). At high glucose concentrations, the metabolism in *K. lactis* and *S. cerevisiae* is respiro-fermentative and thus levels of intra-mitochondrial NADH are lower than when the yeasts are using pyruvate only oxidatively, that is, in accordance with the low expression of the internal dehydrogenase under these conditions.

We have also demonstrated that, under the culture conditions used in this work, *KlNDI1* transcription does not increase with oxygen levels, whereas *S. cerevisiae NDI1* transcription has been reported to be moderately down-regulated under anaerobiosis (Kwast *et al.*, 2002). This result is surely related with the observed prevalence of respiration over fermentation, and *vice versa*, in *K. lactis* and *S. cerevisiae*, respectively (González Siso *et al.*, 2000), and with the need of a rapid adaptation to changing oxygen levels. As proposed by Ter Linde *et al.* (1999), genes with similar aerobic and

anaerobic transcription levels may contribute to this metabolic flexibility, characteristic of facultative yeasts.

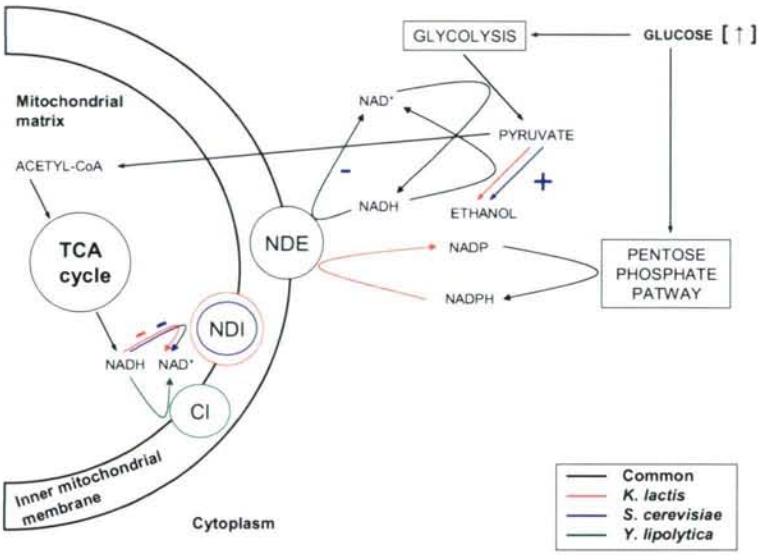

Figure 6: A scheme of the relation between the models of respiro-fermentative metabolism of three yeast species (*Y. lipolytica* that is strictly aerobic, *S. cerevisiae* that is facultative and Crabtree-positive, *K. lactis* that is facultative and Crabtree-negative) and the characteristics of their alternative mitochondrial dehydrogenases. Black lines correspond to elements that are common to the three models, colour lines correspond to elements specific of one or two of the three species as indicated in the Figure. + and – indicate reactions that are activated or inhibited, respectively, when the yeasts grow under high glucose concentrations. CI = complex I, NDI = internal alternative dehydrogenase, NDE = external alternative dehydrogenase.

Our data reveal that the metabolic differences between *S.cerevisiae* and *K. lactis* are more dependent on the characteristics of the external alternative dehydrogenases than on the internal enzymes. Although Klnde1p is in its nucleotide-binding site more similar to the *S. cerevisiae* enzymes than to the *N. crassa* and *S. tuberosum* NADPH dehydrogenases, we have demonstrated that, differently from *S. cerevisiae* whose external dehydrogenases are exclusively NADH-specific (Small and McAlister-Henn, 1998; Luttik *et al.*, 1998), the *K. lactis* homologous enzyme uses NADPH as substrate, *KlNDE1* expression being regulated by the levels of cytosolic NADPH. Without excluding the possibility that a putative second external dehydrogenase could also contribute to this phenotype, the NADPH-oxidizing activity of Klnde1p supports the high activity of the pentose phosphate pathway (Jacoby *et al.*, 1993), allowing the required NADPH/$NADP^+$ turnover. It also explains the ability to grow in glucose of the phosphoglucose isomerase mutant of *K. lactis*. Both events were previously correlated with a high activity of the respiratory chain (González Siso *et al.*, 1996a).

Again, unlike with *S. cerevisiae*, *KlNDE1* transcription is not regulated by the carbon source. *Saccharomyces cerevisiae NDE1* and *NDE2* mRNAs increase after the diauxic shift when the glucose is replaced by ethanol as carbon source (de Risi *et al.*, 1997). The rational is that these enzymes are more important for the oxidative than for the fermentative metabolism (Kwast *et al.*, 2002; Ter Linde *et al.*, 1999). Since mitochondrial alternative external dehydrogenases may compete with alcohol dehydrogenases for the cytosolic NADH (Luttik *et al.*, 1998), the different regulation shown by these enzymes in *S. cerevisiae* and *K. lactis* could be one of the several factors that support the different point of onset of aerobic fermentation, i.e., the Crabtree-positive or negative phenotype.

In conclusion, the results of the work here presented on alternative NAD(P)H dehydrogenases from the yeast *K. lactis* and previous data published on NAD(P)H dehydrogenases from other yeasts led us to postulate that the characteristics of the mitochondrial NAD(P)H dehydrogenases can be directly related to the type of respiro-fermentative metabolism shown by each yeast species (schematized in Fig. 6). Thus, the presence of an alternative internal dehydrogenase, instead of complex I, allows the rapid adaptation of facultative yeasts to the different levels of carbon source and oxygen that occur during growth. Moreover, the ability of the external dehydrogenases to use NADPH in addition to NADH is in correlation with the capacity to metabolize glucose via the pentose phosphate pathway at the expense of glycolysis. Also, their decrease in expression in high glucose concentrations is in correlation with a higher amount of reoxidation of glycolytic NADH by the alcohol dehydrogenases vs. the mitochondria, and therefore with the prevalence of aerobic fermentation and the Crabtree-positive phenotype.

Acknowledgements

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CHAPTER 5

Reoxidation of cytosolic NADPH in *Kluyveromyces lactis*

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SUMMARY

Saccharomyces cerevisiae and *Kluyveromyces lactis* are considered the prototypes of two distinct metabolic models of facultatively-aerobic yeasts: Crabtree-positive/fermentative and Crabtree-negative/respiratory, respectively. Our group had previously proposed that one of the molecular keys supporting this difference lies in the mechanisms involved in the reoxidation of the NADPH produced as a consequence of the activity of the pentose phosphate pathway. It has been demonstrated that a significant part of this reoxidation is carried out in *K. lactis* by mitochondrial external alternative dehydrogenases which use NADPH, the enzymes of *S. cerevisiae* being NADH-specific. Moreover, the NADPH-dependent pathways of response to oxidative stress appear as a feasible alternative that might co-exist with direct mitochondrial reoxidation.

THE REOXIDATION OF CYTOSOLIC NADPH AS A DIFFERENTIAL POINT BETWEEN *Kluyveromyces lactis* AND *Saccharomyces cerevisiae*

Saccharomyces cerevisiae and *Kluyveromyces lactis* are considered the prototypes of two distinct metabolic models of facultatively-aerobic yeasts: Crabtree-positive/fermentative and Crabtree-negative/respiratory, respectively. Although the molecular events supporting these differences have been investigated, they have not been hitherto completely elucidated (for a review see González Siso *et al.* 2000).

Yeasts are able to metabolize glucose by glycolysis or by the pentose phosphate pathway. The pentose phosphate pathway has been reported to be more active in *K. lactis* than in *S. cerevisiae*, e.g., transaldolase activity is about five fold higher in *K. lactis* than in *S. cerevisiae* (Jacoby *et al.*, 1993). The mechanisms involved in the reoxidation of NADPH, produced as a consequence of the operation of the pentose phosphate pathway, were envisaged as the key feature leading to the metabolic differences between *S. cerevisiae* and *K. lactis* (González Siso *et al.*, 1996a). The mutants in the glycolytic phosphoglucose isomerase of *S. cerevisiae* (*pgi1*) and *K. lactis* (*rag2*) are essential tools used to study such mechanisms. While the *S. cerevisiae pgi1* mutant is unable to grow in glucose (Maitra, 1971), the *K. lactis rag2* mutant can (Goffrini *et al.*, 1991). The growth in glucose of the *rag2* mutant (RAG+=resistance to antimycin A in glucose) is dependent on an active mitochondrial respiratory chain since it does not grow in glucose-antimycin A (Goffrini *et al.*, 1991).

When glycolysis is interrupted at the phosphoglucose isomerase step, the glucose 6-phosphate can be re-routed through the pentose phosphate pathway and a

surplus of NADPH is generated in the oxidative reactions of the pathway. In *K. lactis*, a null mutant in the genes encoding subunits of the phosphofructokinase was also able to grow in glucose, but when the gene encoding the pentose phosphate pathway transaldolase was also mutated, this mutant lost its ability to grow in glucose (Jacoby *et al.*, 1993). In *S. cerevisiae*, it has been proposed that the lack of $NADP^+$ blocks the oxidative part of the pentose phosphate pathway, and therefore, glucose utilization by the *pgi1* mutant (Boles *et al.*, 1993). The *K. lactis rag2* mutant grows in glucose, taking advantage of a highly operative pentose phosphate pathway and using, in parallel, a highly efficient system for NADPH reoxidation, directly or indirectly related to the mitochondrial respiratory chain. Although it may seem a paradox, in the *K. lactis rag2* mutant, even glucose fermentation becomes respiration-dependent since the synthesis of pyruvate from C-3 metabolites derived from ribose 5-phosphate is dependent on the oxidative part of the pentose phosphate pathway, and therefore, dependent on the $NADP^+$/NADPH ratio (González Siso *et al.*, 1996a).

Throughout this paper we survey the research done in our laboratory and others, looking for the nature of the molecular mechanisms for cytosolic NADPH reoxidation in *K. lactis*. These mechanisms contribute to the metabolic differences observed between *K. lactis* and *S. cerevisiae*.

WHICH MECHANISMS COULD BE INVOLVED IN THE REOXIDATION OF THE NADPH PRODUCED IN THE PENTOSE PHOSPHATE PATHWAY IN *K. lactis*?

A variety of putative mechanisms involved in the reoxidation of the NADPH produced in the pentose phosphate pathway arose during the design and implementation of this work. A scheme of these pathways is presented in Fig. 1 and their implications on the utilization of glucose by the *K. lactis rag2* mutant are discussed below.

Direct mitochondrial reoxidation by external alternative dehydrogenases

Two observations suggested that in *K. lactis* NADPH reoxidation could be mediated by a cytoplasmic-side mitochondrial NAD(P)H dehydrogenase that would transfer electrons to ubiquinone (González Siso *et al.*, 1996a). The first observation was the observation that growth of the *rag2* mutant in glucose is prevented by a blockade of the mitochondrial respiratory chain after ubiquinone (e.g. by antimycin A) but not before ubiquinone (e.g. by malonate). The second observation was that the activity of the mitochondrial respiratory chain (measured as oxygen consumption and *KlCYC1* expression) is higher when the *rag2* mutant grows in glucose than in fructose,

a monosaccharide that may enter directly in glycolysis without the operation of the pentose phosphate pathway in this mutant. When the respiratory chain is blocked by malonate at the level of the succinate dehydrogenase complex, which passes electrons to ubiquinone, mitochondrial respiration is inhibited but the putative external NAD(P)H dehydrogenase would allow reoxidation of the cytoplasmic NAD(P)H, thus explaining the capacity of the *K. lactis rag2* mutant to grow in the presence of this inhibitor at the expense of fermentation. As explained hereafter, this hypothesis turned out to be correct and was validated by different approaches that finally led us to the cloning and characterization of the corresponding genes (Tarrío *et al.*, 2005).

NADPH provides reducing power to a whole set of metabolic reactions, and therefore, indirect mechanisms which may co-exist with direct mitochondrial NADPH reoxidation could be envisaged. In the following paragraphs each alternative is discussed (Fig. 1). The clues for selecting possible pathways to investigate the actual contribution of each alternative mechanism to NADPH reoxidation in *K. lactis* came from previous experiments undertaken to achieve restoration of the growth in glucose of the *S. cerevisiae pgi1* mutant (Boles *et al.*, 1993). Taking into account the metabolic differences between *S. cerevisiae* and *K. lactis*, especially in relation to the pentose phosphate pathway, the results obtained with the *pgi1* mutant are not directly extrapolable to the *rag2* mutant and therefore each mechanism had to be tested in *K. lactis*.

Indirect mechanisms for NADPH reoxidation

Transhydrogenase and shuttle cycles

Transhydrogenases, which convert NADPH directly into NADH, have not been reported in yeast. However, it has been proposed that pairs of isoenzymes are able to fulfill this task.

Boles *et al.* (1993) transformed the *S. cerevisiae pgi1* mutant with its own genomic library and restored growth in glucose by overproduction of the NAD-glutamate dehydrogenase, thus inducing NADPH-glutamate dehydrogenase. This creates a cyclic "transhydrogenase-like" system that transforms NADPH into NADH and regenerates $NADP^+$ for the pentose phosphate pathway. Dickinson *et al.* (1995) isolated a suppressor of the *S. cerevisiae pgi1* mutation causing simultaneously increased flux through the pentose phosphate pathway and increased NAD^+- and NADPH-dependent glutamate dehydrogenase activities.

Exploring the importance of this mechanism in *K. lactis*, we studied if a similar transhydrogenase-like system could contribute to NADPH reoxidation in the *rag2*

mutant growing in glucose (Becerra *et al.*, 2004). We considered the possibility that the dependence of the *rag2* mutant on the respiratory chain to grow in glucose might be due to the need for reoxidation of a NADH surplus that exceeded the capacity of ethanol fermentation.

Other transhydrogenase-like shuttles have been proposed to operate in the *K. lactis rag2* mutant growing in glucose. Overkamp *et al.* (2002) proposed the existence of a two-compartment (cytoplasm-mitochondria) cycle involving NADPH- and NAD^+-dependent alcohol dehydrogenases. Consistent with measurements of ethanol respiration by mitochondria, these authors found that transcription of *ADH3*, coding for a mitochondrial alcohol dehydrogenase, was extremely low in aerobic glucose-limited chemostat cultures of the wild type strain and induced in the *rag2* mutant. Simultaneously, the activity of an uncharacterized cytosolic NADPH-dependent acetaldehyde reductase was also increased. The *K. lactis* cytosolic alcohol dehydrogenases (acetaldehyde reductases) characterized up-to-date are strictly NAD-dependent (Bozzi *et al.*, 1997).

Verho *et al.* (2002) transformed the *S. cerevisiae pgi1* mutant with *a K. lactis* genomic library and screened for growth on glucose. They found a gene encoding a phosphorylating glyceraldehyde-3-phosphate dehydrogenase that accepts both $NADP^+$ and NAD^+. Although operation of this enzyme in the reverse direction enabled the transformed *pgi1* mutant to oxidize the surplus NADPH, transcription of the gene was not detected in a *K. lactis rag2* mutant grown on glucose, eliminating this possibility.

Mechanisms of response to oxidative stress

Growth in glucose of the *S. cerevisiae pgi1* mutant may be achieved by adding oxidizing agents such as hydrogen peroxide or menadione, thereby causing oxidative stress to yeast cells (Boles *et al.*, 1993). The oxidative stress response of *S. cerevisiae* includes up-regulation of genes encoding for enzymes that use NADPH as cofactor to keep glutathione and thioredoxin reduced (Koerkamp *et al.*, 2002). Therefore, stress mechanisms dependent on NADPH are a metabolic supply of oxidized $NADP^+$ under these conditions (Minard and McAlister-Henn, 2001).

The yeast cells obtain the extra NADPH needed during the stress response by redirecting carbohydrate fluxes to the pentose phosphate pathway at the expense of glycolysis (Godon *et al.*, 1998). The deviation of the glucose 6-phosphate from the glycolysis to the pentose phosphate pathway is precisely what occurs when the *K. lactis rag2* mutant grows in glucose. We then assayed if the NADPH-dependent pathways of defense to oxidative stress were operative in the *K. lactis rag2* mutant growing in glucose. It has been estimated that about 2% of total oxygen uptake

undergoes incomplete reduction to water during mitochondrial respiration and generates ROS (reactive oxygen species: superoxide, hydrogen peroxide and hydroxyl radicals) (Michiels *et al.*, 2002). The existence of a high respiratory rate in the *rag2* mutant (González Siso *et al.*, 1996a) could increase intracellular levels of ROS which would set off the oxidative stress response.

Other enzymes using NADPH

NADPH is consumed in other metabolic pathways such as the biosynthesis of fatty acids, sterols, amino acids and purines, the reduction of oxidized nitrogen sources to ammonia, or the metabolism of certain pentoses. It is also the cofactor of the Old Yellow Enzyme whose physiological role is still unknown (Miranda *et al.*, 1995) but that has been reported to increase during adaptive stress response to hydrogen peroxide in *S. cerevisiae* (Godon *et al.*, 1998).

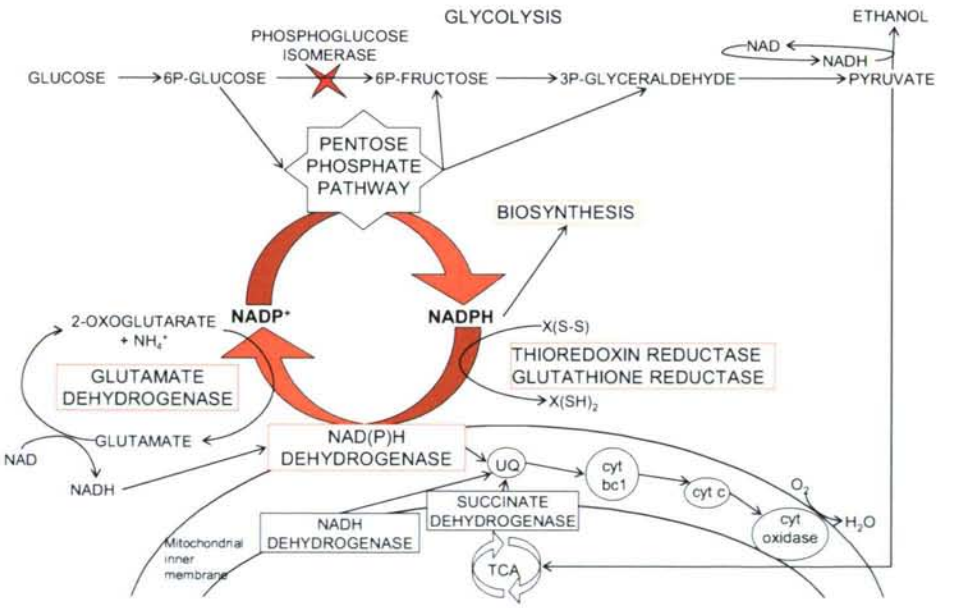

Figure 1: A scheme of the pathways considered during the development of this work as putatively implicated in NADPH reoxidation in the *K. lactis rag2* mutant growing in glucose.

EXPERIMENTAL APPROACH TO THE MECHANISMS INVOLVED IN THE REOXIDATION OF CYTOSOLIC NADPH IN *K. lactis*.

Whether one of the above cited mechanisms, a balance of several or others not envisaged *a priori* account for the growth in glucose of the *K. lactis rag2* mutant is a key question. With this aim, in the last years we performed a screening of induced

molecular mechanisms in the *K. lactis rag2* mutant growing in glucose versus fructose and versus the wild type strain, both at the transcriptional and enzyme activity levels. We also cloned and functionally characterized several *K. lactis* genes encoding alternative mitochondrial dehydrogenases and reductases involved in the oxidative stress response (Becerra *et al.*, 2004; Tarrío *et al.*, 2005, 2004).

For this research, we used for the earlier experiments the *K. lactis* wild type strain NRRL-Y1140 (CBS2359) and the *rag2* mutant strains PM5-2D (Wésolowski-Louvel *et al.*, 1992), and CBS2359 *rag2*::loxP (Steensma and ter Linde, 2001) for recent experiments. Growth of PM5-2D in fructose was considerably worse than in glucose, but was sufficient to allow us to perform some experiments comparing metabolism in both carbon sources. Unlike the case of PM5-2D, the CBS2359 *rag2*::loxP strain is a deletant that is unable to grow in fructose media (Steensma and ter Linde, 2001).

A transcriptome analysis reinforces the role of mechanisms of NADPH consumption and disproves the existence of a transhydrogenase-like cycle.

The general approach was the analysis of the transcriptome of the *K. lactis rag2* mutant PM5-2D growing in glucose versus fructose and versus the wild type strain (Becerra *et al.*, 2004). We hybridized *S. cerevisiae* DNA-arrays with cDNA from *K. lactis* and the heterologous array system proved to be useful to investigate the possible routes to reoxidize the NADPH produced in the pentose phosphate pathway. Selected results were verified by Northern blotting and analyzed more thoroughly by complementary techniques.

Surprisingly, this global transcriptional analysis showed that the mutation in the phosphoglucose isomerase causes in *K. lactis* very little disturbance upon the expression of genes of the pentose phosphate pathway and glycolysis when glucose is the carbon source. We attributed this result to the fact that this yeast, and even the wild type strains, readily uses the pentose phosphate pathway to catabolize glucose (Becerra *et al.*, 2004). This is also supported by a recently published study of metabolic flux analysis in 14 hemi-ascomycetous yeasts, which found that *K. lactis* was among the species with the highest relative pentose phosphate pathway fluxes, whereas *S. cerevisiae* was among those with the lowest (Blank *et al.*, 2005). Therefore, we used as a model to investigate the NADPH reoxidation, the comparison of the transcriptome of the *K. lactis rag2* mutant growing on glucose versus fructose, as differences in intracellular NADPH turnover were notable in these two media (Becerra *et al.*, 2004). In the *K. lactis rag2* mutant, all the glucose must necessarily be metabolized through the glucose 6-phosphate dehydrogenase and following reactions, thus yielding two moles

of NADPH per mol of glucose, whereas fructose may enter directly into glycolysis or into the non-oxidative pentose phosphate pathway, without NADPH production.

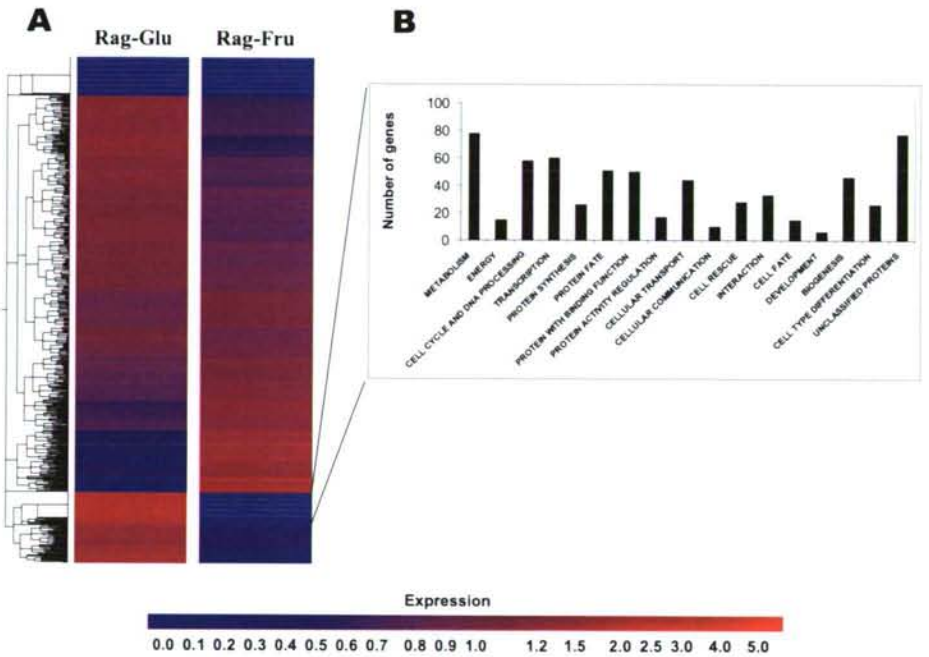

Figure 2: (A) Comparative cluster analysis of the transcriptomes of the *K. lactis rag2* mutant PM5-2D growing in glucose and fructose media. The genes that are over the median of normalized values between the different samples are in red and those below in blue. The relationship between the intensity of the color is proportional to the normalized values as represented on the scale at the bottom of the Figure. (B) Histogram showing the summary of *K. lactis* genes that are overexpressed in glucose versus fructose media classified by functional groups, as defined by the Munich information centre for protein sequences (MIPS) (http://mips.gsf.de/genre/proj/yeast/index.jsp).

The comparative analysis of these two transcriptomes (Fig. 2) corroborated the previously reported (González Siso *et al.*, 1996a) increase in the respiratory metabolism of the *K. lactis rag2* mutant growing in glucose. Among the group of genes showing significantly higher expression in glucose than in fructose, none were outstanding for encoding proteins putatively involved in biosynthetic NADPH consumption, the Old Yellow Enzyme, shuttle or transhydrogenase-like cycles. Specifically, the absence of glutamate dehydrogenase genes from this group was verified by Northern blot with a homologous probe (a partial *KlGDH1* sequence Valenzuela *et al.*, 1995). This result together with the direct measurement of enzyme activities led us to conclude that the glutamate dehydrogenase does not operate as a

transhydrogenase cycle in the *K. lactis rag2* mutant (Becerra *et al.*, 2004). Analysis were performed from cells grown in minimal medium, as transcription of *KlGDH1* was scarcely detected in more complex media.

Interestingly, a moderate increase in transcript levels of some genes involved in the defense against oxidative stress was observed by comparing the transcriptome of the *rag2* mutant in glucose versus fructose. This result reveals the oxidative stress response routes as an interesting alternative for NADPH reoxidation and prompted us to study such routes in *K. lactis*, which were mostly unexplored in this yeast.

Reductases involved in the oxidative stress response and NADPH consumption

Several reductases involved in the cellular defense against oxidative stress are NADPH-dependent, e.g., glutathione reductase and thioredoxin reductase. We also studied if they accounted for the reoxidation of the surplus NADPH produced in the pentose phosphate pathway when the *K. lactis rag2* mutant grows in glucose. This possibility is supported by the observation that a group of genes of the oxidative stress response are transcriptionally activated in the *K. lactis rag2* mutant on glucose (Becerra *et al.*, 2004). In addition, the *K. lactis rag2* mutant seems to be more resistant to the oxidative stress than is the wild type strain. At a fixed concentration of H_2O_2 (2 mM) the treatment time required to reach 50% dead cells is approximately double for the *K. lactis rag2* mutant than for the wild type strain (unpublished results).

To study these systems in *K. lactis*, two *K. lactis* genes homologous to the *S. cerevisiae GLR1* and *TRR1* (encoding glutathione and thioredoxin reductases, respectively) were cloned by the DCbyPCR method (Díaz Prado *et al.*, 2004). The study of the regulation of these genes and their corresponding encoded enzymes showed differences with the *S. cerevisiae* counterparts (Tarrío *et al.*, 2004). First, *K. lactis GLR1* does not respond to H_2O_2 treatment, neither by changes in mRNA transcription nor by enzyme activity modulation, whereas *S. cerevisiae* glutathione reductase is an oxidative stress defense inducible enzyme and its gene a Yap1p-target (Grant et al., 1996a). Second, *KlTRR1* is the single gene encoding thioredoxin reductase present in *K. lactis*, whereas *S. cerevisiae* contains two isoforms of the protein, cytosolic and mitochondrial, encoded by two genes, *TRR1* and *TRR2*, respectively (Grant, 2001). Differently to *KlGLR1* but similar to the *S. cerevisiae TRR1/TRR2* genes (Ross *et al.*, 2000), *KlTRR1* transcription and enzyme activity are induced after H_2O_2 treatment, a Yap1p binding site being functional in the *KlTRR1* promoter (Tarrío *et al.*, 2004). Both *KlGLR1* and *KlTRR1* show predicted mitochondrial-targeting signals. It has been recently demonstrated that *S. cerevisiae GLR1* gives rise

to two locations of the protein, cytosolic and mitochondrial, as a function of the selection of the transcription start site (Outten and Culotta, 2004). A similar mechanism could occur in the *K. lactis* genes. We are now studying the subcellular location of the glutathione and thioredoxin reductases of *K. lactis*.

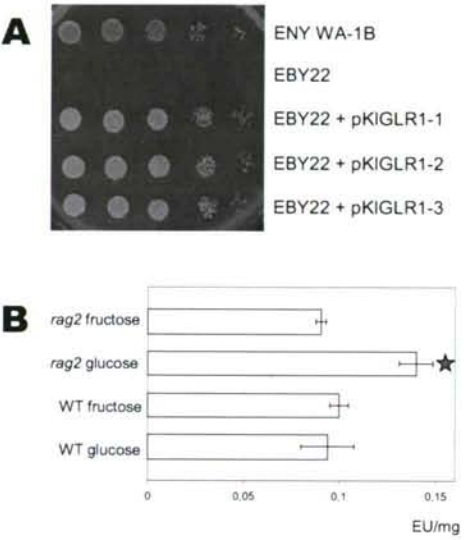

Figure 3: Role of the *K. lactis* glutathione reductase in NADPH reoxidation. (A) Growth in glucose of the *S. cerevisiae pgi1* mutant EBY22 (Boles *et al.*, 1993) transformed with an episomic plasmid bearing the *KlGLR1* gene (three independent transformants), and the corresponding wild type strain ENY-WA-1B (Boles *et al.*, 1993). (B) Glutathione reductase activity in the *K. lactis rag2* mutant PM5-2D and the wild type strain in glucose and fructose media. The asterisk indicates significant differences with the others at the 95% confidence level.

The expression of the *KlGLR1* gene under its own promoter in an episomic plasmid completely restores the growth on glucose of the *S. cerevisiae pgi1* mutant; the transformants grow as well as the wild type strain (Fig. 3A). Comparison of glutathione and thioredoxin reductase activities in the *K. lactis rag2* mutant PM5-2D growing in glucose versus fructose, in the absence of H_2O_2 treatment, showed only small differences (Tarrío *et al.*, 2004). Unexpectedly, thioredoxin reductase activity was even greater in fructose. Glutathione reductase activity showed a small increase in the *rag2* mutant in glucose (Fig. 3B); this increase in activity was also observed when the strain CBS2359 *rag2*::loxP was compared to the wild type using controlled fermentor cultures in glucose (unpublished data). This suggests a putative role for this enzyme in cytosolic NADPH reoxidation, complementary to the external mitochondrial dehydrogenases described below. The glutathione reductase, in *S. cerevisiae* and other organisms, has been reported to regulate the activity of glucose 6-phosphate dehydrogenase by means of the control of the $NADP^+$/NADPH ratio through the glutathione redox interconversion (López-Barea *et al.*, 1990). Perhaps in *K. lactis*, this

control is performed at two points: the glutathione reductase, as in *S. cerevisiae*, but also the external mitochondrial dehydrogenases, the differential point. The role of the *K. lactis* glutathione reductase in the redox metabolism is being the subject of further research.

The external alternative dehydrogenases of *K. lactis* mitochondria use NADPH

Mitochondrial alternative dehydrogenases are a family of proteins of the inner membrane that can oxidize NAD(P)H and reduce ubiquinone without proton-pumping activity. These are rotenone-insensitive and single polypeptide enzymes whose only prosthetic group is flavin adenine dinucleotide (FAD) and that can oxidize either cytosolic (external enzymes) or matrix substrates (internal enzymes). They are absent in mammals but found in plants and fungi (Kerscher, 2000; Josep-Horne *et al.*, 2001).

The suggestion of the existence of a cytoplasmic-side mitochondrial NAD(P)H dehydrogenase in *K. lactis* was our first attempt at interpreting the reoxidation of cytosolic NADPH in the *rag2* mutant (González Siso *et al.*, 1996a), although the knowledge about alternative mitochondrial dehydrogenases in yeast was scarce at that time. Consistent with our hypothesis, it was later demonstrated, that mitochondria of *K. lactis* can oxidize cytosolic NADPH (Overkamp *et al.*, 2002), whereas *S. cerevisiae* mitochondria cannot (Small and McAlister-Henn, 1998; Luttik *et al.*, 1998). Moreover, isolated mitochondria from a *K. lactis rag2* mutant showed higher respiration rates than mitochondria from a wild type strain; respiration increase followed exogenous addition of NADPH and NADH and, therefore, argued in favor of the existence of an external dehydrogenase with dual cofactor specificity (Overkamp *et al.*, 2002).

The first step in the study of the external alternative dehydrogenases of *K. lactis* mitochondria in our laboratory was the cloning of the genes. When we published the hypothesis on the presence and function of such dehydrogenases in *K. lactis* (González Siso *et al.*, 1996a), no genes of similar enzymes from yeast or related organisms had been characterized. The identification of the counterpart genes of *S. cerevisiae* was published two years later (Small and McAlister-Henn, 1998; Luttik *et al.*, 1998), where the authors investigated two ORFs of unknown function from the *Saccharomyces* genome sequence database which showed similarity to the gene encoding the internal alternative dehydrogenase (*NDI1*). Identification of *NDE1* and *NDE2* genes made *S. cerevisiae* the first eukaryote in which the genes encoding external NADH dehydrogenases had been identified. One year later, the characterization of the genes of the single external enzyme of the yeast *Yarrowia lipolytica* and of the first alternative enzyme from the filamentous fungus *Neurospora*

crassa was published (Kerscher *et al.*, 1999; Melo *et al.*, 1999). When the random partial sequences (RSTs) of the *K. lactis* genome were made available by *Génolevures* (http://cbi.labri.u-bordeaux.fr/Genolevures), we found in this database a very useful tool to isolate a full-length gene with significant similarity to the *S. cerevisiae NDE1* and *NDE2* genes; this *K. lactis* gene was named *KlNDE1* and its functional analysis is described in Tarrio *et al.* (2005).

The primary sequence of the putative protein Klnde1p showed two conserved glycine-rich motifs for nucleotide binding, one for NAD(P)H and the other for FAD; we tried to infer from this sequence if one of these motifs was able to bind NADPH or if it was NADH-specific, but it was not clear (Tarrio *et al.*, 2005). It has been proposed that in the glycine-rich consensuses (GXGXXG) of the NADPH-binding sites, the third Gly is generally replaced by Ser, Ala, or Pro, and also a conserved negatively charged residue (Glu or Asp) downstream is substituted generally by Asn, thus missing the negative charge and avoiding its unfavorable interaction with the also negatively-charged 2´-phosphate of NADPH (Lesk, 2001). The external mitochondrial dehydrogenases of the yeasts *S. cerevisiae* and *Y. lipolytica* have been reported to be active with NADH and not with NADPH (Small and McAlister-Henn, 1998; Luttik *et al.*, 1998; Kerscher *et al.*, 1999). More recently, fungal (*N. crassa*) and plant (*Solanum tuberosum*) genes encoding external mitochondrial NADPH dehydrogenases have been identified (Melo *et al.*, 2001; Carneiro *et al.*, 2004; Michalecka *et al.*, 2004). When we aligned these NADH and NADPH dehydrogenases and Klnde1p with the aim of comparing the nucleotide-binding motifs, we observed that these two features typical of the NADPH-binding glycine-rich consensuses were both present only in the first motif of the calcium-dependent protein Nde1p from *N. crassa* (Melo *et al.*, 2001), but not in the others, independently of whether the protein actually used NADH, NADPH or both (Fig. 4).

```
NcNDE1          KLALNPRRGGPKNLPILEIFLDDDDSEEKKKHKEKPRLVILGGGWGSVALLKELNPDDYH 172
StNDB1          ------------------VVEQNQPESKKK-----RVVVLGTGWGGTSFLKDVDISSYD 80
KlNDE1          --------------------PQIPQAATFANGSPRKTLVVLGTGWGSVSLLKHLDTSLYN 125
ScNDE1          --------------------TQVPQSDTFPNGSKRKTLVILGSGWGSVSLLKNLDTTLYN 137
ScNDE2          --------------------KQVPQSTAFANGLKKKELVILGTGWGAISLLKKLDTSLYN 122
NcNDE2          --------------------PQVEPD------PSKKTLVVLGTGWGSVSLLKKLDTEHYN 137
YlNDE1          --------------------DQLPAD------PSKKTLVVLGSGWGSVSFLKKLDTSNYN 137
                                     :              :*:** ***. ::**.::   *.
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Figure 4: A fragment of a Clustal W of NADH and NADPH external alternative dehydrogenases (NDE), showing the first glycine-rich motif for coenzyme binding and also the conserved Asp downstream. Nc= *Neurospora crassa*, St=*Solanum tuberosum*, Yl=*Yarrowia lypolytica*, Kl=*Kluyveromyces lactis*, Sc=*Saccharomyces cerevisiae*.

The ability of Klnde1p to bind NADPH was then verified from experimental results (Tarrío *et al.*, 2005). One important clue was the fact that the *S. cerevisiae pgi1* mutant was able to grow on glucose when transformed with an episomic plasmid expressing the *KlNDE1* gene from its own promoter region, although the growth rate of the transformants was considerably lower than that of the wild type strain (Fig. 5A). We also analyzed the expression of the *KlNDE1* gene and the results obtained supported the ability of Klnde1p to bind NADPH. Thus, Northern blot analysis showed a low but significant increase of *KlNDE1* expression in the *rag2* mutant in glucose versus fructose and the wild type strain. The disappearance of this transcriptional induction of *KlNDE1* after treatment with hydrogen peroxide, i.e., after induction of the NADPH-consuming defense mechanisms against oxidative stress and concomitant reduction of intracellular NADPH levels, suggested a role for Klnde1p in cytosolic NADPH reoxidation (Tarrío *et al.*, 2005). An important confirmation came from the isolation of mitochondria of the *S. cerevisiae nde1* mutant expressing the *K. lactis NDE1* gene and the evidence that such mitochondria were able to exogenously oxidize added NADPH (Tarrío *et al.*, 2005), as shown in Fig. 5B, although at a slightly slower rate than that reported for *K. lactis* wild type mitochondria (Overkamp *et al.*, 2002). This lower rate was attributed either to the above described regulation of *KlNDE1* or to the possible existence of a second alternative NADPH dehydrogenase (Tarrío *et al.*, 2005). The isolated mitochondria of the *S. cerevisiae nde1* mutant expressing the *K. lactis NDE1* gene also exogenously oxidized added NADH (Tarrío *et al.*, 2005), as shown in Fig. 5B.

The recent availability through the Génolevures database of the complete sequence of the *K. lactis* genome revealed the existence of another putative alternative external dehydrogenase (we named it Klnde2p); this one is similar to the *N. crassa* external calcium-dependent dehydrogenase and we are now studying its role in cytosolic NADPH reoxidation. However, the recent construction in our laboratory of a *K. lactis nde1rag2* double deletion mutant in the CBS2359 background and the finding that this strain grows scarcely not only in glucose but also in a series of carbon sources tested (unpublished results) support the hypothesis that Klnde1p plays a major role in the reoxidation of the NADPH produced in the pentose phosphate pathway in the *rag2* mutant. In fact, the double mutation is practically lethal after a few cell divisions. Therefore, it seems *a priori* that the participation of Klnde2p in the reoxidation of the NADPH produced in the pentose phosphate pathway may be less important than that of Klnde1p. In spite of this, the regulatory network of the two external alternative dehydrogenases would be interesting to study, not only from the point of view of the

NADPH reoxidation but also to investigate the precise metabolic role of this class of enzymes in the different organisms in which they have been described.

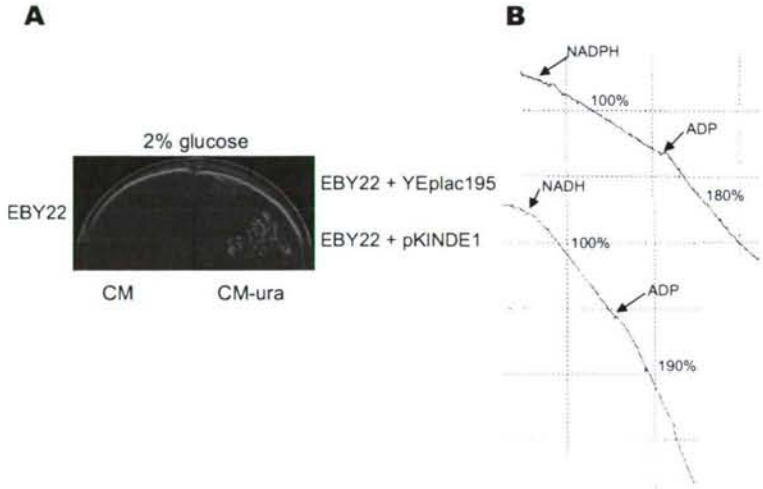

Figure 5: NADPH oxidation by the *K. lactis* external alternative mitochondrial dehydrogenase Klnde1p. (A) Growth in glucose of the *S. cerevisiae pgi1* mutant EBY22 (Boles *et al.*, 1993) transformed with an episomic plasmid, empty (YEplac195) or expressing the *KlNDE1* gene. (B) Oxygen consumption rate, following exogenous addition of NADPH and NADH, by isolated mitochondria of the *S. cerevisiae nde1* mutant transformed with an episomic plasmid expressing the *KlNDE1* gene (pKlNDE1). Control experiments showed that the oxygen consumption rate by isolated mitochondria of the untransformated *S. cerevisiae nde1* mutant was undetectable with NADPH and 1.4-fold lower with NADH as substrate.

The alternative dehydrogenases of yeast as a metabolic differential clue

Apart from *K. lactis*, at present the genomes of three other yeasts have been completely sequenced by the Génolevures consortium (*Candida glabrata*, *Debaromyces hansenii* and *Yarrowia lipolytica*), these sequences being available in the Génolevures database. We performed a comparative study of selected genes of these yeasts showing high similarity with those of the alternative dehydrogenases of *S. cerevisiae* and *N. crassa*; this study allowed us to reach some conclusions and to propose some generalizations. Fig. 6 shows a phylogram of the putative alternative dehydrogenases of the four yeasts whose genomes have been completely sequenced by Génolevures.

A first surprising observation was that, although it has been reported that a single external enzyme confers alternative NADH:ubiquinone oxidoreductase activity in *Y. lipolytica* and that the mitochondria of this yeast do not exogenously oxidize added NADPH (Kerscher *et al.*, 1999), there is another sequence (YALI0E05599g) in the genome of this yeast showing similarity to alternative dehydrogenases, specifically to those of *N. crassa* and *K. lactis* containing a calcium-binding domain (Fig. 6). These

three enzymes show in the first nucleotide-binding domain the conserved aspartate in the NADH-binding proteins changed by a basic residue, as proposed for NADPH-binding domains (Lesk, 2001). The protein from *N. crassa* has been reported to bind NADPH (Melo *et al.*, 2001).

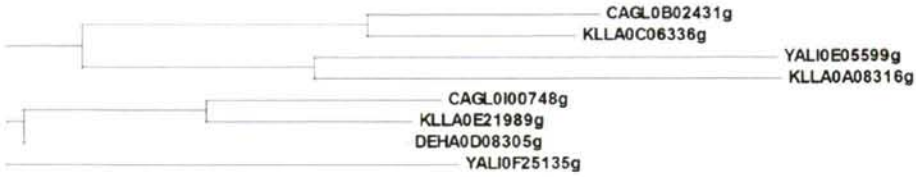

Figure 6: Phylogram (Clustal W at http://www.ebi.ac.uk) of the putative mitochondrial alternative dehydrogenases found in the complete yeast genomes available from the Génolevures databases. The upper secondary branch corresponds to internal enzymes (CAGL0B02431g, KLLA0C06336g), the following secondary branch to enzymes with a calcium binding domain (YALI0E05599g, KLLA0A08316g), and the rest to external enzymes without a calcium binding domain (CAGL0I00748g, KLLA0E21989g, DEHA0D08305g, YALI0F25135g). KLLA = *Kluyveromyces lactis*, CAGL= *Candida glabrata*, YALI= *Yarrowia lipolytica*, DEHA = *Debaromyces hansenii*.

The comparative study also revealed that, unlike what occurs in plants and *N. crassa*, in the yeast genomes sequenced so far, complex I and the alternative internal dehydrogenase are not present simultaneously. *Yarrowia lipolytica* and *D. hansenii* are strictly aerobic yeasts (Gancedo and Serrano, 1989) that contain complex I; *C. glabrata*, *K. lactis* and also *S. cerevisiae* are facultative yeasts that replace complex I by an alternative internal dehydrogenase. It seems that the strictly aerobic yeasts prefer complex I, which is composed of many subunits but yields a higher P/O ratio than the more versatile single polypeptide alternative enzyme, which is preferred by the facultative yeasts adapted to fermentation, as proposed in Tarrio *et al.*, (2005).

Moreover, the genome of *C. glabrata* contains two genes encoding putative alternative dehydrogenases, one predicted as external and the other as internal by similarity (Fig. 6). As previously reported for *S. cerevisiae* (Luttik *et al.*, 1998) and *K. lactis* (Tarrio *et al.*, 2005) also the mitochondrial-targeting signal (predicted using the program MitoProt, http://ihg.gsf.de/ihg/mitoprot.html) of the putative external alternative dehydrogenase of *C. glabrata* is longer than that of the internal enzyme. Probably, these signal peptides contain the information that determines the cytosolic or matrix orientation of the proteins in the inner mitochondrial membrane.

Further study of the alternative dehydrogenases of these and other yeasts is needed; it will contribute not only to our understanding of the biology of mitochondrial coenzyme reoxidation but also to elucidate the molecular basis of the respiro-fermentative metabolism in yeast. For example, competition for cytosolic NADH formed

in glycolysis between mitochondria oxidative systems and alcohol dehydrogenases has been proposed as a relevant factor in the occurrence of aerobic fermentation and the Crabtree effect (Luttik *et al.*, 1998; Tarrío *et al.*, 2005). The external dehydrogenases of *S. cerevisiae* show, in the presence of high glucose concentrations, a transcriptional down-regulation (De Risi *et al.*, 1997) that is absent in *KlNDE1* (Tarrío *et al.*, 2005) and that may be related to an increased flow of glycolytic NADH through the alcohol dehydrogenases and the increase in the fermentative metabolism observed in *S. cerevisiae* versus *K. lactis*. Moreover, in a group of 14 hemi-ascomycetous yeasts a correlation has been found between the flux through the pentose phosphate pathway and the activity of the tricarboxylic acid cycle (Blank *et al.*, 2005). Although merely speculative for now, in yeasts when the pentose phosphate pathway is supported by alternative external mitochondrial dehydrogenases able to reoxidize NADPH, a respiratory metabolism predominates.

OUTLOOK

At the present state of knowledge, we propose that the mitochondrial alternative external dehydrogenases of *K. lactis* represent the main pathway for the reoxidation of cytosolic NADPH. This supports a high activity of the pentose phosphate pathway and allows growth of the *rag2* mutant in glucose. The activity of the glutathione reductase could also contribute to the adaptation of the *rag2* mutant to glucose catabolism based exclusively on the pentose phosphate pathway, thus explaining the more respiratory lifestyle and the higher resistance to oxidative stress of this strain. Interesting questions about the interplay of the pentose phosphate pathway activity, the respiratory chain, the alternative mitochondrial dehydrogenases, the defense mechanisms against oxidative stress and the differences of respiro-fermentative metabolism in yeasts are open to further study.

Acknowledgements

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CHAPTER 6

Oxidative stress response and cytosolic NADPH turnover in the respiratory yeast *Kluyveromyces lactis*

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SUMMARY

The respiratory yeast *Kluyveromyces lactis* shows a higher relative flux of glucose through the pentose phosphate pathway than the fermentative yeast *Saccharomyces cerevisiae*. The *K. lactis* phosphoglucose isomerase mutant (*rag2*) is forced to metabolize the glucose uniquely through the oxidative pentose phosphate pathway and shows an increased respiratory chain activity. In this work we have studied the oxidative stress response involved in the NADPH reoxidation and its relationship with the respiro-fermentative metabolism, using yeasts as model. We have proved that *K. lactis* is more resistant to peroxide-mediated oxidative stress than *S. cerevisiae* and that the *K. lactis rag2* mutant is more resistant than the wild type strain. Increased levels in the rag2 mutant of two enzymatic activities, catalase and NADPH-dependent glutathione reductase, support this resistance. In *K. lactis* glucose 6-phosphate dehydrogenase is positively regulated by oxygen and correlated with glutathione reductase. Glutathione reductase, even if overexpressed, is not enough to allow growth on glucose of the *rag2* mutant when the mitochondrial reoxidation of cytosolic NADPH is blocked by antimycin A or by oxygen deprivation. Similarly, peroxide-mediated oxidative stress, which induces thioredoxin reductase but not glutathione reductase or glucose 6-phosphate dehydrogenase, does not restore the growth on glucose-antimycin A of the *rag2* mutant.

INTRODUCTION

The fermentative yeast *Saccharomyces cerevisiae* is being traditionally used as a model eukaryote for studying cellular responses to oxidative stress. Since increased reactive oxygen species (ROS) formation during respiratory versus fermentative growth has been proposed (Cabiscol *et al.*, 2000), the respiratory yeast *Kluyveromyces lactis* is an interesting alternative model for studying oxidative stress. There is a previous report on NADPH-dependent reductases in *K. lactis* (Tarrío *et al.*, 2004) suggesting that the oxidative stress response, hitherto mostly unexplored, does not follow exactly the same pattern described for *S. cerevisiae*.

The relative flux of glucose through glycolysis or the pentose phosphate pathway and the concomitant mechanisms used for NADPH reoxidation is a key point of the different respiro-fermentative metabolism shown by *S. cerevisiae* and *K. lactis* (Tarrío *et al.*, 2006). Under fully aerated conditions, *S. cerevisiae* shows much lower pentose phosphate pathway and tricarboxylic acid cycle (TCAc) fluxes than *K. lactis* (Blank *et al.*, 2005). The mutants in the glycolytic phosphoglucose isomerase (*pgi1*

from *S. cerevisiae* and *rag2* from *K. lactis*) are forced to catabolize all the glucose 6-phosphate through the oxidative reactions of the pentose phosphate pathway. The *pgi1* mutants cannot grow on glucose due to their inability to reoxidize the surplus of NADPH produced in the pentose phosphate pathway (Boles *et al.*, 1993). In contrast, the *rag2* mutants can grow on glucose since *K. lactis* can reoxidize this NADPH via a mitochondrial external alternative dehydrogenase (González Siso *et al.*, 1996a; Tarrío *et al.*, 2005), being the counterpart enzymes from *S. cerevisiae* NADH-specific (Luttik *et al.*, 1998; Small and Mc-Alister-Henn, 1998).

In *K. lactis*, the NADPH-dependent pathways of response to oxidative stress have been proposed as an additional mechanism for providing $NADP^+$ to the pentose phosphate pathway (Tarrío *et al.*, 2006). The *rag2* mutant in glucose shows an increased activity of the respiratory chain (González Siso *et al.*, 1996a) that could increase intracellular levels of ROS (Møller, 2001) which would set off the oxidative stress response. In fact, a moderate transcriptional induction of some genes involved in the defense against oxidative stress was observed in the *rag2* mutant in glucose (Becerra *et al.*, 2004). Also, the NADPH-dependent glutathione reductase activity was found to be increased in the *rag2* mutant in glucose (Tarrío *et al.*, 2006). In this work we have demonstrated that the *K. lactis rag2* mutant is more resistant to oxidative stress than the wild type strain and we have inquired about the enzymatic bases of this phenomenon.

The oxidative stress response of *S. cerevisiae* includes the up-regulation of the reductases of glutathione and thioredoxin that obtain the required NADPH in the cytoplasm mainly from the glucose 6-phosphate dehydrogenase of the pentose phosphate pathway (Koerkamp *et al.*, 2002; Grant, 2001). In this work we have examined the activity of these three NADPH-related enzymes in *rag2* and wild type *K. lactis* strains in response to the addition of peroxides and to different activities of the respiratory chain (aerobic versus hypoxic conditions), and we have studied the putative role of these oxidative stress defense reactions in the reoxidation of the NADPH from the pentose phosphate pathway in *K. lactis*. We have also studed as a control under the same conditions the activity of catalase, a typical oxidative stress defense enzyme not involved in NADPH reoxidation and that catalyzes the dismutation of H_2O_2 into H_2O and O_2.

The aim of this work was to study, from an enzymatic point of view, the relationship between respiratory/fermentative metabolism in yeasts and the response to oxidative stress. The relative contribution of the oxidative stress defense reactions versus the mitochondrial external alternative dehydrogenases in supplying $NADP^+$ to the pentose phosphate pathway was also studied.

MATERIAL AND METHODS

Strains, media and culture conditions

The following *K. lactis* strains were used: the wild type NRRL-Y1140 (*MATa*, ATCC8585, CBS2359), MW190-9B (*Mata lac4-8 uraA Rag*$^+$), the *rag2* (phosphoglucose isomerase) mutants PM5-2D (*MATa uraA1-1 metA1-1 argA1-1 trpA1-1 rag2-1*) (Wésolowski-Louvel *et al.*, 1992) and CBS2359 *rag2*::loxP (Steensma and Ter Linde, 2001).

The following *S. cerevisiae* strains, supplied by Euroscarf (http://www.uni-frankfurt.de/fb15/mikro/euroscarf/col_index.html), were used: BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ*) and Y12737 (BY4742 *YPL091w::kanMX4*). The ORF YPL091w corresponds to the *GLR1* gene.

The *S. cerevisiae* strains ENY.WA-1B (*MATa ura3-52 leu2-3,112 trp1-289 MAL2-8^c MAL3 SUC3*) and the phosphoglucose isomerase mutant EBY22 (ENY.WA-1B *pgi1Δ::TRP1*) (Boles *et al.*, 1993) were also used.

Growth and handling of yeasts were carried out according to standard procedures (Kaiser *et al.*, 1994). The yeast cells were cultivated, unless otherwise stated, in Erlenmeyer flasks at 30°C and 250 rpm in the synthetic complete medium CM (Zitomer and Hall, 1976) or the dropout medium CM-ura (without uracil) containing 2% glucose (w/v). The media for PM5-2D were supplemented with methionine and arginine (Sigma Co.) up to a concentration of 40 mg/L. The flasks were filled with 40% volume of culture medium. Solid growth media also contained 1.5% agar (w/v).

The viability after treatment with H_2O_2 was studied as follows: the *K. lactis* wild type and PM5-2D strains were grown in liquid CM-medium as described above up to OD_{600}=0.6 and then shifted to the same medium with different concentrations of H_2O_2 from 0.4 to 50 mM. Samples were taken at pre-defined time intervals up to 120 minutes and serial dilutions were plated on solid CM-medium and incubated at 30°C for two days. The grown colonies were counted and percentage of viable cells was estimated in reference to a control without H_2O_2 treatment.

Growth in presence of the respiratory inhibitor antimycin A (Sigma Co.) or the oxidant agents tBOOH (tert-butyl-hydroperoxide) (Sigma Co.) or H_2O_2 was analyzed on solid CM or YPD medium (2% bactopeptone, 1% yeast extract, 2% glucose, w/v) supplemented with 5 μM antimycin A or 1-5 mM tBOOH or H_2O_2. Cells from overnight or stationary phase (48-h growth) Erlenmeyer cultures in CM or YPD were diluted up to OD_{600}=0.35-1, as indicated in each case, and then several serial 1:10 dilutions were performed. Drops of 0.01 mL of the dilutions were put on the solid media and incubated for 2-5 days at 30°C.

To generate oxidative stress conditions, unless otherwise stated, the cells of the wild type and *rag2 K. lactis* strains were grown in liquid CM-medium as described above up to OD_{600}=0.6 and then shifted to the same medium with either 0.4 mM H_2O_2 or 0.4 mM tBOOH for 30 minutes. The experiments of the 2^2 experimental design were performed under the same conditions but varying the concentration of the oxidant and the time of exposure as indicated.

To study the effect of the oxygen availability on enzyme activities, the yeast cells were grown in a 2-L vessel fermentor (Biostat MD; Braun-Biotech, Germany) in YPD medium (2% bactopeptone, 1% yeast extract, 0.5% glucose, w/v) supplemented with 0.002% ergosterol (w/v) (Sigma Co.). The initial working volume of the culture was 2 L and temperature was maintained at 30°C. The culture was agitated at a speed of 300 rpm. The air flow was 240 L/h (2 v.v.m.). Nitrogen (99.95% pure; Carburos Metálicos, Spain) was bubbled at a constant pressure of 0.9 bars. Dissolved oxygen was measured with a polarographic electrode. The oxygen electrode was calibrated prior to inoculation of the cultures by equilibration with air (full scale, relative pO_2 = 100%) and nitrogen (zero, relative pO_2 = 0%). Cells of the wild type and *rag2*::loxP *K. lactis* strains were grown overnight under air flow up to OD_{600}=0.3-0.9 when a sample was taken (o/n O_2) and air was immediately replaced with nitrogen. Two samples were taken thereafter at time intervals of 3 and 6 hours (3h N_2 and 6h N_2). Then nitrogen was replaced with air and a fourth sample was taken 3 hours thereafter (3h O_2). Glucose consumption was followed through reducing sugar determination by the procedure of Bernfeld (1951).

Determination of enzyme activities

For the determination of enzyme activities, protein extracts were prepared as described in Tarrio *et al.* (2004): The cells from 400 mL of the Erlenmeyer cultures or 200 mL of the fermentor cultures were collected by centrifugation and resuspended in 1 mL of buffer A (0.2 M Tris-HCl pH=7.8, 0.3 M $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol v/v, chemicals

from Sigma Co.) per gram of wet weight. Cells were broken by vortexing with glass beads (1.5 mm diameter) in 20-s pulses. The supernatant obtained after a centrifugation at 8000 rpm for 15 minutes was immediately used. All steps were performed in cold.

Protein concentration was measured by the method of Bradford (1976) (Bio- Rad) using bovine seroalbumin (Sigma Co.) as a standard.

Glutathione reductase and thioredoxin reductase activities were determined as described in Smith *et al.* (1988) and Holmgren and Björnstedt (1995), respectively. Enzyme units (E.U.) are defined as μmol TNB (5'-thionitrobenzoic acid)/ minute × mg protein, under assay conditions.

Glucose 6-P-dehydrogenase activity was determined by the method of Kuby and Noltmann (1966). Enzyme Units (E.U.) are expressed in terms of micromoles of NADPH formed per milligram of protein per minute, in the assay conditions.

Catalase was determined by the method of Aebi (1984). Enzyme units (E.U.) are defined as the decrease of the concentration of H_2O_2 expressed in micromoles per milligram of protein per minute, in the assay conditions.

Protein extracts were prepared from several independent cultures of each experiment. Up to four enzyme activity measurements were carried out for each protein extract and the results averaged.

Statistical data analysis and experimental design

Data are expressed as mean ± standard error (S.E.). The statistical significance of differences found between means was evaluated by the ANOVA and Multiple Comparisons Tests performed by the program StatGraphics Plus (Manugistics).

The StatGraphics Plus software was also used to create factorial experimental designs and to analyze the data obtained.

Other procedures

Standard procedures for manipulation of nucleic acids were essentially those of Sambrook *et al.* (1989). *Escherichia coli* DH-10B was used for plasmid amplification. Yeast transformation was performed using the lithium acetate procedure (Ito *et al.*, 1983).

RESULTS

The phosphoglucose isomerase (*rag2*) mutant of *Kluyveromyces lactis* is more resistant to H_2O_2 than the wild type strain

In Fig. 1 the percentage of viable cells (determined from the number of colony forming units as described in Materials and Methods) remaining after different treatments of *S. cerevisiae* wild type, *K. lactis* wild type and *rag2* (PM5-2D) strains with H_2O_2 are shown. The lowest concentration of H_2O_2 examined, 0.4mM, influenced viability in *S. cerevisiae* but not in *K. lactis*. At higher H_2O_2 concentrations (≥ 1mM), a sharper decrease of viability along the time of exposure was observed for the wild type than for the *rag2* strain.

Significant fractions (about 2%) of oxygen uptake are converted to ROS by the mitochondrial respiratory chain (Inoue *et al.*, 2003) and NADPH plays a role in detoxification of ROS as a cofactor of several enzymes (Temple *et al.*, 2005). Therefore, the increased resistance to oxidative stress of the *rag2* mutant could be related to an adaptative response to the increased mitochondrial respiration and/or NADPH reoxidation rates, both concomitant to the obligated metabolism of glucose

through the oxidative pentose phosphate pathway (González Siso *et al.*, 1996a). In support of this hypothesis is the fact that *K. lactis* shows higher resistance to oxidative stress than *S. cerevisiae* (Fig. 1) being *K. lactis* more respiratory and with a higher flow of glucose through the pentose phosphate pathway than *S. cerevisiae* (Blank *et al.*, 2005). Then, this hypothesis is further considered in light of the subsequent experiments described bellow.

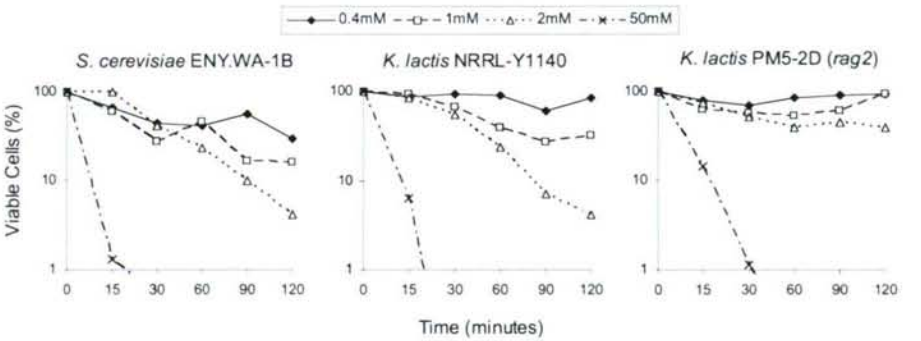

Figure 1: Resistance to oxidative stress of yeasts with different respiro-fermentative metabolism. Viable Cells (percentage of colony forming units) of the *S. cerevisiae* (ENY.WA-1B), *K. lactis* wild type (NRRL-Y1140) and *rag2* (PM5-2D) strains after treatment with different H_2O_2 concentrations as described in Materials and Methods. Results are the average of two independent experiments.

Enzymatic responses to oxidative stress in the *K. lactis rag2* mutant *versus* the wild type strain

The NADPH-dependent glutathione reductase and thioredoxin reductase catalyze reactions for the regeneration of two of the most abundant and important cellular antioxidants, being glucose 6-phosphate dehydrogenase the main source of NADPH in the cytoplasm for these reductases (Grant, 2001). We studied the levels of these three NADPH-related enzyme activities in the wild type and *rag2*::loxP strains under normal or oxidative stress conditions created by treatment with tBOOH as described in Materials and Methods. The activity of catalase, that does not supply $NADP^+$ to the pentose phosphate pathway, was also studied under the same conditions as a control to test whether the higher activity of the respiratory chain of the *rag2* mutant in glucose was effectively causing an oxidative stress to the cell. The results are presented in Fig. 2 and show differences between the *rag2* and wild type strains.

Catalase activity, which is induced by tBOOH in both strains, is higher in the *rag2*::loxP mutant than the wild type strain under normal conditions (Fig. 2). These results support the hypothesis of an induction of the general oxidative stress response

in the *K. lactis rag2* mutant growing in glucose by affecting reactions not directly involved in NADPH reoxidation.

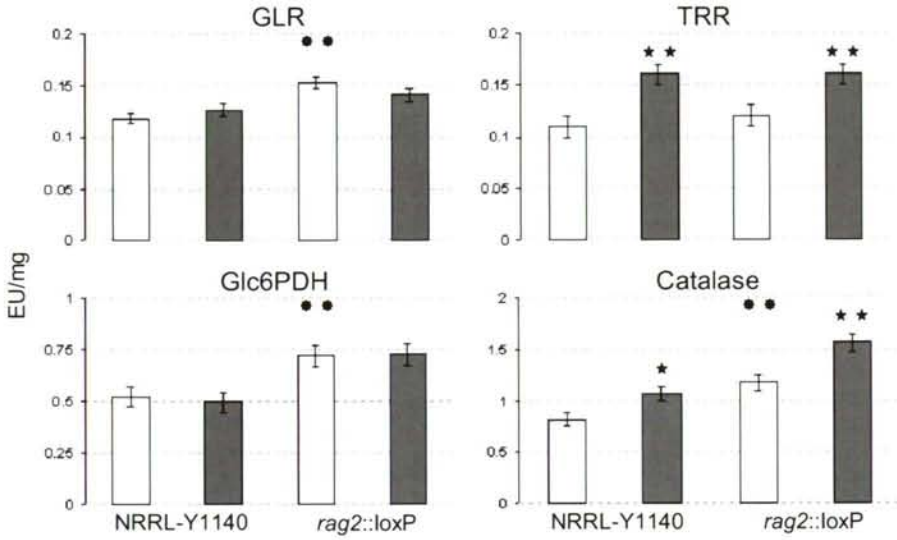

Figure 2: Glutathione reductase (GLR), thioredoxin reductase (TRR), glucose 6-phosphate dehydrogenase (Glc6PDH) and catalase activities in the *K. lactis* wild type (NRRL-Y1140) and *rag2*::loxP mutant strains under normal (white bars) and oxidative stress (gray bars) conditions created by addition of 0.4mM tBOOH as described in Materials and Methods. Results are the average of two independent cultures each analyzed at least in duplicate, $4 \leq n \leq 7$. Five point stars indicate significant differences between normal and oxidative stress conditions for the same strain and enzyme activity. Eight point stars indicate significant differences between strains for the same enzyme activity under normal conditions. One star indicates the 95% confidence level; two stars indicate the 99% confidence level.

The results presented in Fig. 2 also show that glutathione reductase activity (but not thioredoxin reductase activity) is higher in the *rag2*::loxP mutant grown in glucose under normal conditions than in the wild type strain. In both strains, the treatment with 0.4 mM tBOOH for 30 minutes significantly increased thioredoxin reductase activity, but not glutathione reductase activity. Similar conclusions regarding these two enzyme activities were previously obtained using the *rag2* mutant PM5-2D and H_2O_2 as oxidant agent (Tarrío *et al.*, 2004; 2005). It is very likely that the activity of catalase, which is increased in the *rag2* mutant versus the wild type, by catalyzing the dismutation of the H_2O_2 formed as a by-product of the respiratory metabolism, causes that thioredoxin reductase is not induced in the *rag2* mutant under normal conditions.

Moreover, glucose 6-phosphate dehydrogenase activity was not induced in *K. lactis* (neither wild type nor *rag2*::loxP strains) after the treatment with 0.4 mM tBOOH for 30 minutes and was lower in the wild type than in the *rag2*::loxP strain (Fig. 2). We also measured this enzymatic activity in the wild type and *rag2* PM5-2D strains after a

treatment with 0.4 mM H_2O_2 for 30 minutes (data not shown) reaching a similar conclusion.

In K. lactis glutathione reductase and glucose 6-phosphate dehydrogenase are indeed not induced by oxidative stress

The *K. lactis* NADPH-related response to peroxide-mediated oxidative stress described above is different from that reported for the conventional yeast *S. cerevisiae* in which glutathione reductase, thioredoxin reductase and glucose 6-phosphate dehydrogenase belong to the H_2O_2–stimulon (Godon *et al.*, 1998). To test if the lack of induction by peroxides of *K. lactis* glutathione reductase and glucose 6-phosphate dehydrogenase was a consequence of a different sensitivity or mild conditions of the treatments, a full factorial experimental design of the type screening 2^2 was performed to study the combined influence of oxidant agent concentration and time of exposure over these two enzyme activities produced by the *K. lactis* wild type and *rag2* strains. Since similar results were obtained with tBOOH on *rag2*::loxP as with H_2O_2 on PM5-2D, we analyzed only the second case. The experimental domain defined by the range of variables whose influence was studied and the corresponding coded values are presented in Table I. The results (experimental points and predicted surface responses) are represented in Fig. 3 and confirm that the NADPH-related response to peroxide-mediated oxidative stress in *K. lactis* does not affect the activity of the enzymes glucose 6-phosphate dehydrogenase and glutathione reductase, as explained below.

Table I: Experimental domain showing the correspondence between natural and coded values of the two variables investigated in the 2^2 full factorial experimental designs.

Coded value	$[H_2O_2]$ (mM)	Time (min)
-1	0.4	15
0	1.2	67.5
1	2	120

Glutathione reductase activity did not vary significantly with concentration of the oxidant or time of exposure in the experimental domain studied for any of the two strains (p-values of the effects and lack of fit of the model > 0.05), i.e., in *K. lactis* glutathione reductase is actually not induced by H_2O_2–treatment. Similarly, mouse liver glutathione reductase is not induced by oxidative stress (Raza *et al.*, 2002).

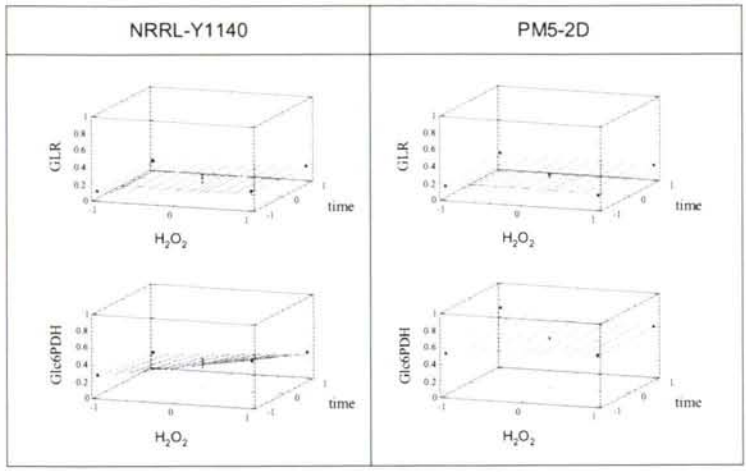

Figure 3: Predicted response surfaces obtained from the 2^2 full factorial experimental design performed according to the experimental domain defined in Table I and in the culture conditions described in Materials and Methods. The central point (0,0) was studied in triplicate (three independent cultures) at random mode, the interval between maximum and minimum experimental values is indicated by a line perpendicular to the surface. The black points represent the experimental results at the four corners. Glutathione reductase (GLR) and glucose 6-phosphate dehydrogenase (Glc6PDH) are expressed in E.U. H_2O_2 concentration and time are expressed in values coded as described in Table I.

As with glutathione reductase, the glucose 6-phosphate dehydrogenase activity of the *rag2* strain did not vary significantly with concentration of the oxidant or time of exposure in the experimental domain studied (p-values of the effects and lack of fit of the model > 0.05). The wild type strain showed a glucose 6-phosphate dehydrogenase activity (Glc6PDH) that increased significantly (p-value=0.02) with H_2O_2–concentration ($[H_2O_2]$) but decreased significantly (p-value=0.04) with time of exposure (t), the effect of the interaction between the two factors ($[H_2O_2]$ t) being non significant (p-value=0.15). The equation of the model is (lack of fit = 0.5) Glc6PDH (E.U.) = 0.33 + 0.11 $[H_2O_2]$ -0.08 t, in which $[H_2O_2]$ and t are in coded values. The effects of $[H_2O_2]$ and t on glucose 6-phosphate dehydrogenase activity are low as inferred from their coefficients and the response surface (Fig. 3).

Besides the different response to peroxide-mediated oxidative stress of these two enzymes versus thioredoxin reductase, a different regulatory mechanism may also influence the transcription of the corresponding genes. A binding site for the transcriptional factor Yap1p, directing oxidative stress induction of many genes, is functional in the *KlTRR1* promoter (Tarrio *et al.*, 2004), whereas apparently there are not Yap1p binding sites in the 500 bp upstream regions of the *KlGLR1* or *KlZWF1* genes using TFSEARCH (http://www.cbrc.jp/htbin/nph-tfsearch). Transcription of the *KlGLR1* gene is not induced by H_2O_2–treatment (Tarrio *et al.*, 2004).

On the other hand, the induction of glutathione reductase activity in the *K.lactis rag2* mutant in glucose could be the response to an increased production of superoxide anion by the more active respiratory chain shown by this strain (González Siso *et al.*, 1996a; Møller, 2001). To test this possibility, *K. lactis* glutathione reductase activity was also quantified in the wild type strain to test if the levels of the *rag2* mutant were achieved in response to the superoxide anions generated by the redox cycling agent menadione added at a concentration of 1mM during 30 minutes. However, as with tBOOH and H_2O_2, the treatment with menadione did not increase significantly glutathione reductase activity in the wild type strain (0.13±0.04 versus 0.12±0.01 with menadione, n=4).

In *S. cerevisiae*, it has been recently demonstrated that factors that help to regenerate reduced glutathione in the mitochondria are crucial in preventing damage during exposition to high oxygen concentrations, being superoxide anion the key damaging agent (Outten *et al.*, 2005).

Once the role of the oxidative stress in the induction of glutathione reductase and glucose 6-phosphate dehydrogenase activities in the *rag2* mutant in glucose was excluded, two new questions arose. Which is the actual mechanism of induction and which is the role of the increased glutathione reductase activity in the resistance of the *rag2* mutant to oxidative stress and/or in the reoxidation of the NADPH from the pentose phosphate pathway?

Effect of oxygen concentration on the activity of oxidative stress response enzymes in *K. lactis* wild type and *rag2* strains

To answer the first question, we hypothesize whether the mechanism/s inducing glutathione reductase and glucose 6-phosphate dehydrogenase activities in the *rag2* mutant growing in glucose could be related to its higher respiratory metabolism. To test this hypothesis, *K. lactis* wild type and *rag2*::loxP strains were grown in controlled fermentor cultures performing the air-nitrogen-air shift described in Materials and Methods in order to vary the oxygen availability and the activity of the respiratory chain; the influence of the resulting aerobic and hypoxic conditions on the activity of glutathione reductase, glucose 6-phosphate dehydrogenase but also thioredoxin reductase and catalase was studied (Fig. 4). The reason to examine the four enzymes in the two strains was that, although we expected *a priori* that the *rag2* mutant was unable to grow under hypoxia since it does not grow on glucose-antimycin A because it needs an active respiratory chain when growing in glucose to reoxidize the NADPH of the pentose phosphate pathway by an external alternative dehydrogenase (González Siso *et al.*, 1996a), we also considered the possibility of an induction under hypoxia of

enzymes of the oxidative stress response using NADPH that could allow the *rag2* mutant to grow. In support of this possibility was the reported increase under hypoxia of transcription of some *S. cerevisiae* genes involved in the oxidative stress response (Causton *et al.*, 2001; Dirmier *et al.*, 2002).

There is no induction of NADPH-consuming oxidative stress defence enzymes in the rag2 mutant under hypoxia

Fig. 4 shows that, as expected *a priori*, the wild type strain is able to grow under the hypoxic conditions used in this work, while the *rag2* mutant is not. The possibility of an induction under hypoxia of enzymes of the oxidative stress response using NADPH sufficient enough to allow the *rag2* mutant to grow was thus rejected.

The hypoxic induction of an oxidative stress response enzyme was observed only for the catalase of the wild type strain after 6 hours of growth under nitrogen (Fig. 4). The re-oxygenation of the wild type hypoxic cells led to a decrease of catalase activity down to the initial values (Fig. 4), which is in accordance with the reported inhibition of the catalase activity in *Kluyveromyces marxianus* under increased oxygen partial pressure (Pinheiro *et al.*, 2002).

The activity of the NADPH-related enzymes glucose 6-phosphate dehydrogenase and glutathione reductase even diminished after the shift from air to nitrogen, although the decrease was less pronounced in the *rag2* mutant than in the wild type strain. Thus, in the case of glucose 6-phosphate dehydrogenase, after 6 hours of nitrogen bubbling, the activity of the wild type was about 30% of initial values in aerated conditions whereas the activity of the *rag2* was about 70% of initial values in aerated conditions (Fig. 4). Also, in the case of glutathione reductase, after 6 hours of nitrogen bubbling, the activity of the wild type was about 60% of initial values in aerated conditions whereas the activity of the *rag2* was about 75% of initial values in aerated conditions (Fig. 4). The initial values in aerated conditions were not recovered after re-oxygenation of the hypoxic cultures. Thioredoxin reductase activity decreased after the shift to hypoxia in the wild type but not in the *rag2* strain (Fig. 4). Although this might be a mechanism of the *rag2* mutant to gain some extra NADPH reoxidation when the alternative mitochondrial dehydrogenases are inactive, this extra NADPH reoxidation is not enough to allow hypoxic growth in glucose of the *rag2* strain.

Glucose 6-phosphate dehydrogenase in K. lactis is regulated by oxygen

Under aerobic conditions, levels of glucose 6-phosphate dehydrogenase activity were similar in *rag2* and wild type strains. Under hypoxia this activity was higher in the *rag2* mutant (Fig. 4). This is likely because when oxygen availability diminishes, the wild type strain diverts glucose to the glycolysis at the expense of the pentose phosphate pathway, while the *rag2* mutant cannot do this. The *rag2* mutant can

ferment glucose to ethanol but it needs the oxidative part of the pentose phosphate pathway to bypass the phosphoglucose isomerase reaction and convert the glucose 6-phosphate to intermediates that enter into glycolysis (González Siso *et al.*, 1996a).

Levels of glucose 6-phosphate dehydrogenase activity in cells overnight grown under fully aerated conditions (o/n O_2 samples) show a good positive correlation with dissolved oxygen levels in both strains (Fig. 5A). This correlation explains why in some experiments performed in Erlenmeyer cultures (Fig. 2), and contrary to previous reports (Becerra *et al.*, 2004), we found a higher glucose 6-phosphate dehydrogenase activity in the *rag2* mutant than in the wild type strain both cultured under similar conditions. The presumably reduced oxygen availability in these Erlenmeyer cultures may cause concomitant reduced flow of glucose through the pentose phosphate pathway in the wild type strain but not in the *rag2* mutant. Glutathione reductase activity, however, is higher in the *rag2* mutant than in the wild type strain both under hypoxic and aerobic conditions (Fig. 4), thus corroborating the results obtained in Erlenmeyer cultures (Fig. 2). Also catalase activity is higher in the *rag2* mutant than in the wild type strain except after 6 hours of nitrogen bubbling when there is an increase of this activity only in the wild type strain (Fig. 4).

Glucose 6-phosphate dehydrogenase and glutathione reductase activities are positively correlated in K. lactis

Glutathione reductase, in *S. cerevisiae* and other organisms, has been reported to regulate the activity of glucose 6-phosphate dehydrogenase by means of the control of the $NADP^+$/NADPH ratio through the glutathione redox interconversion (López-Barea *et al.*, 1990). Fig. 5B shows that in *K. lactis*, both in wild type and *rag2* strains, there is a significant positive correlation between glutathione reductase and glucose 6-phosphate deshydrogenase activities, whereas Fig. 5C shows that there is no strong correlation between thioredoxin reductase and glucose 6-phosphate deshydrogenase activities. These results suggest a role of glutathione reductase in pentose phosphate pathway-NADPH reoxidation.

Moreover, Fig. 5B also shows that the glutathione reductase activity is higher in the *rag2* mutant than in the wild type strain when levels of glucose 6-phosphate dehydrogenase activity are similar. The results shown in Fig. 5 suggest that the induction of the glutathione reductase in the *K. lactis rag2* mutant, different from glucose 6-phosphate dehydrogenase, is not directly driven by culture oxygen levels, since culture oxygen levels are the same when glucose 6-phosphate dehydrogenase activity is similar in both strains. Likewise, similar pentose phosphate pathway-NADPH levels may be inferred from a similar glucose 6-phosphate dehydrogenase activity and then NADPH levels cannot cause the induction of the glutathione reductase activity in

the *K. lactis rag2* mutant. The cause of this induction remains to be identified and will be the subject of further research in our laboratory.

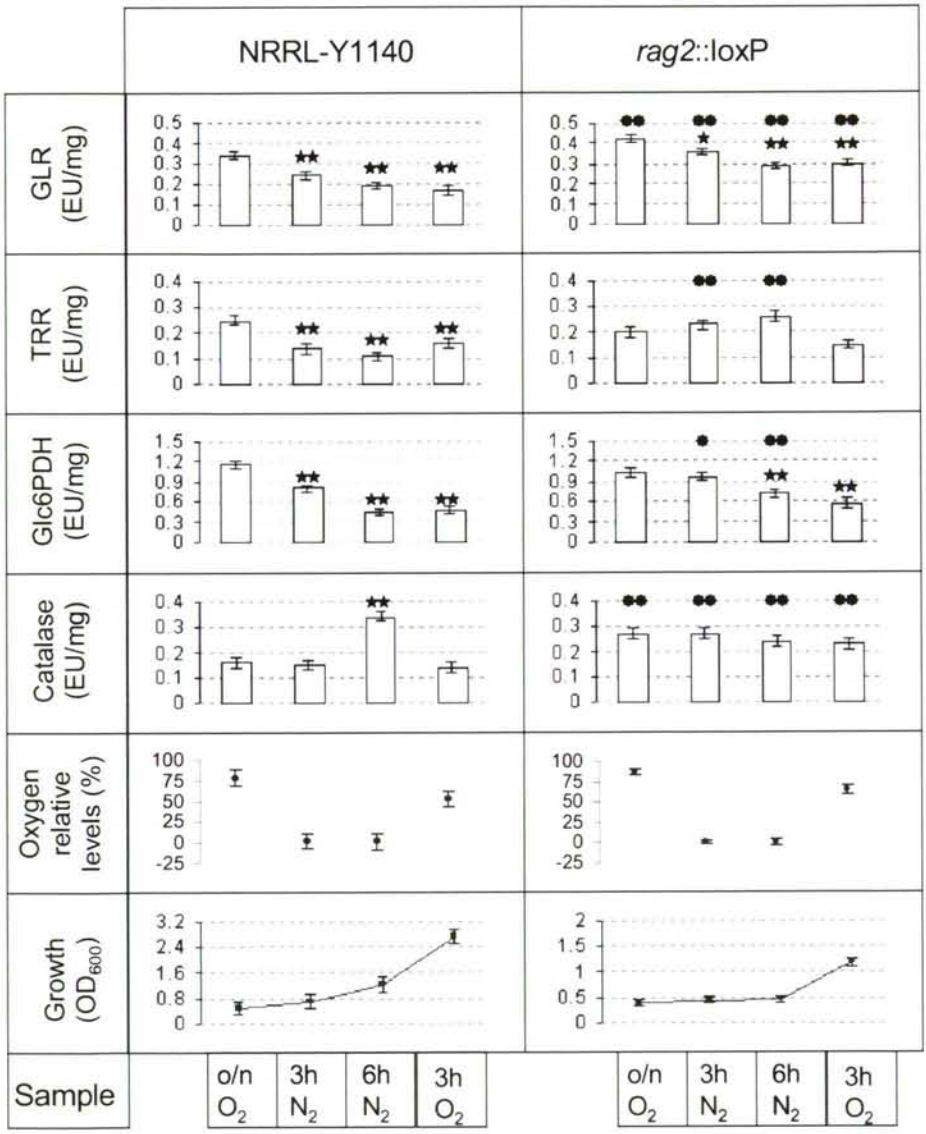

Figure 4: Growth, dissolved oxygen, glutathione reductase (GLR), thioredoxin reductase (TRR), glucose 6-phosphate dehydrogenase (Glc6PDH) and catalase activities in the *K. lactis* wild type (NRRL-Y1140) and *rag2*::loxP mutant strains in fermentor cultures under the shift air-nitrogen-air in the conditions described in Materials and Methods. Glucose consumption was less than 10% of initial values. pH was maintained at 6-7 without addition of acid or alkali. Results are the average of three independent cultures, $3 \leq n \leq 11$. Five point stars indicate significant differences of each enzyme activity with the initial aerated condition (o/n O_2) for the same strain. Eight point stars indicate significant differences of each enzyme activity between strains under the same conditions. One star indicates a 95% confidence level; two stars indicate a 99% confidence level.

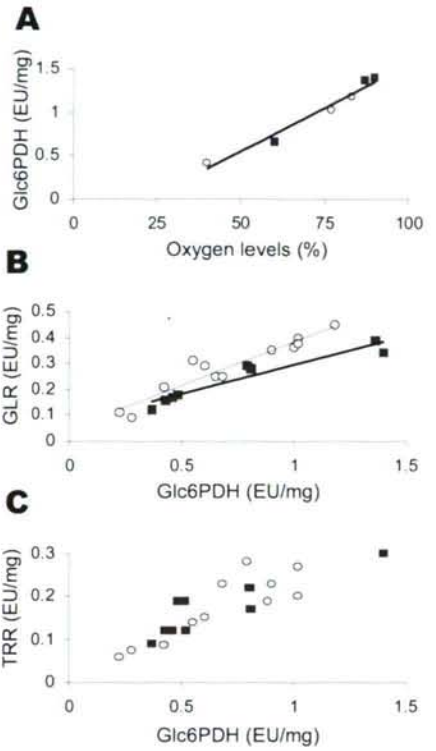

Figure 5: (A) Correlation between glucose 6-phosphate dehydrogenase activities and dissolved oxygen levels under the initial aerated conditions (o/n O_2 samples) from the cultures of Fig. 4 (R^2=0.97); black squares correspond to the *K. lactis* wild type and white circles to the *rag2*::loxP mutant. (B) Correlation between glutathione reductase and glucose 6-phosphate dehydrogenase activities in the *K. lactis* wild type (black squares, R^2=0.91) and *rag2*::loxP mutant (white circles, R^2=0.91) strains in the fermentor cultures of Fig. 4. (C) Correlation between thioredoxin reductase and glucose 6-phosphate dehydrogenase activities in the *K. lactis* wild type (black squares, R^2=0.77) and *rag2*::loxP mutant (white circles, R^2=0.76) strains in the fermentor cultures of Fig. 4.

Does overexpression of *KlGLR1* cause an increase in oxidative stress tolerance?

We have shown in the previous sections that *K. lactis* glutathione reductase was not induced by treatment with peroxides or menadione and that it was higher in the *rag2* mutant than the wild type strain under normal conditions (Fig. 2-4). Therefore, to assay if this activity was related to the higher resistance to oxidative stress of the *rag2* mutant, the effect of the overexpression of the *KlGLR1* gene encoding *K. lactis* glutathione reductase on the resistance to peroxide-mediated oxidative stress was studied in both *S. cerevisiae* and *K. lactis*.

The *KlGLR1* gene (nucleotides -1155 to +994), was cloned into the polylinker of the episomic plasmid YEplac195. With the resulting construction the *S. cerevisiae Δglr1*

mutant Y12737 was transformed, and growth on plates of YPD medium supplemented with tBOOH was studied. The transformants expressing the *KlGLR1* gene grew, in the presence of 5 mM tBOOH, appreciably better than the untransformed *S. cerevisiae* *Δglr1* mutant and even better than the corresponding wild type strain (Fig. 6A).

We have previously reported (Tarrío *et al.*, 2004) that the overexpression of *KlGLR1* from an episomic plasmid induces 8-fold the glutathione reductase activity of the *K. lactis* strain MW190-9B. Fig. 6B shows that this overexpression induces the resistance to oxidative stress in *K. lactis* by a small amount, thus, the transformants expressing the *KlGLR1* gene grew, in the presence of tBOOH or H_2O_2, slightly better than the untransformed *K. lactis* wild type strain.

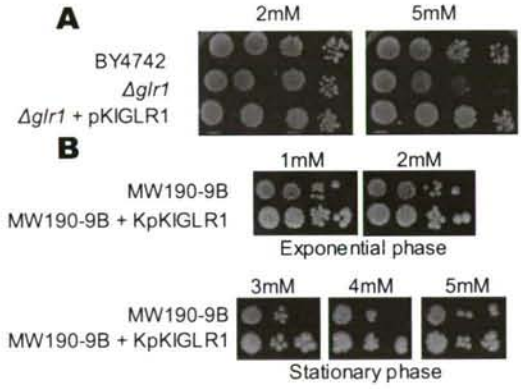

Figure 6: Increased resistance to oxidative stress of *S. cerevisiae* and *K. lactis* by overexpression of *K. lactis* glutathione reductase. (A) Increased resistance to oxidative stress created by tBOOH (2 and 5 mM) of the *S. cerevisiae Δglr1* mutant transformed with an episomic plasmid expressing the *KlGLR1* gene (pKlGLR1) encoding *K. lactis* glutathione reductase. BY4742 is the wild type strain. Cultures of strains were grown into stationary phase and adjusted to an initial OD_{600}=1 before spotting onto appropiate plates. Plates were incubated at 30°C for four days. (B) Increased resistance to oxidative stress created by tBOOH (1 and 2 mM) or H_2O_2 (3, 4 and 5 mM) of the *K. lactis* wild type strain MW190-9B transformed with an episomic plasmid expressing the *KlGLR1* gene (KpKlGLR1) encoding *K. lactis* glutathione reductase. Cultures of strains were grown into exponential phase or stationary phase and adjusted to an initial OD_{600}=1 before spotting onto appropriate plates. Plates were incubated at 30°C for five days.

Therefore, in spite of the fact that *K. lactis* glutathione reductase is not induced by peroxide mediated oxidative stress, when the level of this activity is increased, the yeast cells (both *S. cerevisiae* and *K. lactis*) became more resistant to oxidative stress. This may be likely what occurs in the *K. lactis rag2* mutant, i.e., the higher resistance to oxidative stress of the *K. lactis rag2* mutant is supported by higher levels of glutathione reductase activity.

Does overexpression of *KlGLR1* or *KlTRR1* cause an increase in pentose phosphate pathway-NADPH reoxidation that restores the growth on glucose-antimycin A of the *K. lactis rag2* mutant?

In *K. lactis* the involvement of glutathione reductase in the reoxidation of the NADPH produced in the pentose phosphate pathway may be inferred from the results revealed in the previous sections of this work showing that there are increased levels of this activity in the *rag2* mutant versus the wild type strain in glucose (Fig. 2 and 4) and that there is a significant correlation between the glutathione reductase and glucose 6-phosphate deshydrogenase activities in both wild type and *rag2* strains (Fig. 5B).

Differently from *S. cerevisiae*, the external alternative mitochondrial dehydrogenase of *K. lactis* use NADPH (Tarrio *et al.*, 2005). In the presence of antimycin A, NADPH reoxidation by external mitochondrial alternative dehydrogenases is blocked. To analyze the relative importance of glutathione reductase versus mitochondrial external alternative dehydrogenase in reoxidation of the pentose phosphate pathway-NADPH, we tested if the overexpression of the glutathione reductase restored the growth on glucose-antimycin A of the *K. lactis rag2* mutant.

We have previously proved that the overexpression of the *KlGLR1* gene restores the growth on glucose of the *S. cerevisiae pgi1* mutant (Tarrio *et al.*, 2006). As shown in Fig. 7A, in the presence of antimycin A the result is the same, this fact suggests that the oxidation of the cytosolic NADPH generated by the pentose phosphate pathway may be carried out by glutathione reductase at an exclusive cytosolic level in *S. cerevisiae*. In order to increase glutathione reductase activity, the *K. lactis rag2* mutant PM5-2D was transformed with the episomic plasmid expressing the *KlGLR1* gene (KpKlGLR1) described in (Tarrio *et al.*, 2004) and growth on glucose-antimycin A was tested. The overexpression of the *KlGLR1* gene did not promote significant growth of the *K. lactis rag2* mutant on glucose-antimycin A (Fig. 7B) with glucose concentrations of 2% or 0.2%. Therefore, the role of glutathione reductase in NADPH reoxidation in the *K. lactis rag2* mutant appears to be less important than the role of the external mitochondrial alternative dehydrogenase.

We have reported previously (Tarrio *et al.*, 2005) that the gene encoding the mitochondrial alternative external dehydrogenase *KlNDE1* is transcriptionally induced in the *rag2* mutant in glucose, this induction being removed by addition of 0.4mM H_2O_2. As shown in the previous sections of this work, differently from glutathione reductase, the activity of the NADPH-consuming thioredoxin reductase is induced by peroxides (Fig. 2), then we decided to test a putative role of thioredoxin reductase in the reoxidation of the pentose phosphate pathway-NADPH in *K. lactis* under this oxidative stress conditions.

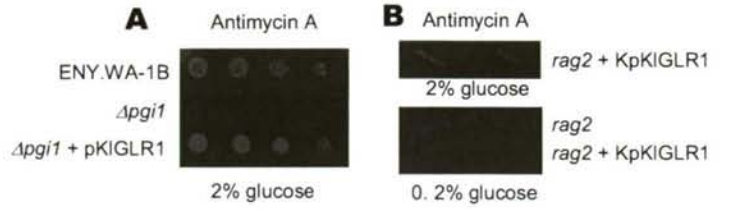

Figure 7: Growth on glucose-antimycin A media of *S. cerevisiae* and *K. lactis* wild type and phosphoglucose isomerase mutant strains with induced oxidative stress response pathways (A) Growth on glucose (CM) with antimycin A of the *S. cerevisiae pgi1* mutant transformed with an episomic plasmid expressing the *KlGLR1* gene encoding *K. lactis* glutathione reductase (pKlGLR1). ENY.WA-1B is the wild type strain. Initial OD_{600}=0.35; two days of growth at 30°C. (B) Growth on glucose (CM) with antimycin A of the *K. lactis rag2* mutant transformed with an episomic plasmid expressing the *KlGLR1* gene encoding *K. lactis* glutathione reductase (KpKlGLR1). Initial OD_{600}=1; six days of growth at 30°C.

To induce the NADPH-consuming thioredoxin reductase the cells of the *K. lactis rag2*::loxP strain were successively grown on plates of solid YPD medium supplemented with 0.4, 1, 2, 5 and 10 mM tBOOH and then growth on glucose-antimycin Å was tested. The *K. lactis rag2* mutant was unable to grow on glucose-antimycin A (with concentrations of 2% or 0.2% glucose) even when the pathways of response to peroxide mediated oxidative stress were induced (not shown). In contrast, in *S. cerevisiae* it has been reported (Boles *et al.*, 1993) that adding oxidant agents to the culture medium the growth on glucose of the *pgi1* mutant was restored.

Since, as demonstrated in this section, the *K. lactis rag2* mutant is unable to grow on glucose-antimycin A with induced thioredoxin reductase or glutathione reductase activities, the mitochondrial external alternative dehydrogenases seem to be the main mechanism for reoxidation of the surplus of pentose phosphate pathway-NADPH in the *K. lactis rag2* mutant both under normal and oxidative stress conditions.

DISCUSSION

The results presented in this work show that there is a relationship between a high respiratory metabolism and the resistance to oxidative stress in eukaryotic cells, using yeasts as model. Thus, with glucose as carbon source, the respiratory *K. lactis* is more resistant to oxidative stress than the fermentative *S. cerevisiae* (Fig. 1). Similarly, the *K. lactis rag 2* mutant, characterized by an increased activity of the respiratory chain (González Siso *et al.*, 1996a), is more resistant to oxidative stress than the wild type strain (Fig. 1). We have demonstrated that this higher resistance of the *rag 2* mutant is supported by higher levels of activity of glutathione reductase and catalase

(Fig. 2 and 6). Also in *S. cerevisiae* glutathione reductase and catalase provide overlapping defences for protection against hydrogen peroxide (Grant *et al.*, 1998).

The mitochondrial electron transport chain has been reported to be a major site of ROS production (Møller, 2001), specifically the external alternative dehydrogenases are a source of superoxide (Fang and Beattie, 2003). Therefore, the higher oxygen consumption of the *rag2* mutant in glucose related to the use of the external alternative NADPH-dehydrogenase (González Siso *et al.*, 1996a), probably by increasing ROS production in the mitochondrial respiratory chain, induces catalase activity, which is in accordance with the increase of this activity upon addition of peroxides (Fig. 2). The mechanism that induces glutathione reductase activity in the *rag2* mutant in glucose is not clear at present. A direct effect of hydroperoxides, superoxide anions, oxygen and NADPH being discarded (Fig. 2, 3 and 5).

The global consideration of the results presented in this work and others previously published also show that the metabolic interplay between oxidative stress response and cytosolic NADPH turnover is different in respiratory yeasts, using *K. lactis* as a model, from fermentative yeasts, using *S. cerevisiae* as a model.

In *K. lactis*, showing a high relative flux of glucose through the pentose phosphate pathway (Blank *et al.*, 2005), the glucose 6-phosphate dehydrogenase is regulated by oxygen (Fig. 5A) and the NADPH from the pentose phosphate pathway can be reoxidized under normal conditions by two main pathways: the external alternative mitochondrial dehydrogenase (Tarrio *et al.*, 2005) and the glutathione reductase (Fig. 5B and 7A). The external alternative mitochondrial dehydrogenase is an essential overflow mechanism for the reoxidation of the surplus of pentose phosphate pathway-NADPH when the *rag2* mutant grows in glucose. Although the glutathione reductase contributes to regenerate $NADP^+$ for the pentose phosphate pathway, this enzymatic activity, even if overexpressed, is not enough to allow growth in glucose of the *rag2* mutant when the mitochondrial reoxidation of cytosolic NADPH is blocked by antimycin A or by oxygen deprivation (Fig. 4 and 7B). Similarly, peroxide-mediated oxidative stress, which induces thioredoxin reductase but not glutathione reductase or glucose 6-phosphate dehydrogenase (Fig. 2 and 3), does not restore the growth in glucose of the *rag2* mutant when the respiratory chain is blocked by antimycin A. The relative importance of the two systems for cytosolic NADPH reoxidation in *K. lactis* is in agreement with the situation in plants. It has been reported that the external mitochondrial NADPH dehydrogenase from plants has the major capacity for NADPH reoxidation if compared to the cytoplasmic pool of cytosolic NADPH-utilizing enzymes (Michalecka *et al.*, 2004).

It seems that in *S. cerevisiae*, which shows a low relative flux of glucose through the pentose phosphate pathway (Blank *et al.*, 2005) and whose external alternative mitochondrial dehydrogenases are NADH-specific (Luttik *et al.*, 1998; Small and Mc-Alister-Henn, 1998), the glutathione reductase and perhaps other cytosolic systems are mainly in charge of the reoxidation of the NADPH of the pentose phosphate pathway (López-Barea *et al.*, 1990; Pócsi *et al.*, 2004). In the *pgi1* mutant all the glucose is metabolized through the pentose phosphate pathway, then NADPH availability exceeds the capacity of these cytosolic systems and the growth in glucose is impaired due to $NADP^+$ depletion (Boles *et al.*, 1993). Oxidative stress induces the NADPH-dependent response pathways glucose 6-phosphate dehydrogenase, glutathione and thioredoxin reductases (Koerkamp *et al.*, 2002; Grant, 2001) and restores growth in glucose of the *pgi1* mutant (Boles *et al.*, 1993). The same restoration occurs when the genes of the *K. lactis* glutathione reductase (Fig. 7A) and external alternative mitochondrial dehydrogenase (Tarrío *et al.*, 2005) are overexpressed in the *S. cerevisiae pgi1* mutant.

We therefore propose that the diverse patterns of respiratory and fermentative metabolism are related to differences in the oxidative stress response through variations in the activity of the pentose phosphate pathway and the mechanisms used for NADPH reoxidation. Further research with other yeast species is needed to generalize this proposal.

Acknowledgements

We thank Dr. M. Wésolowski-Louvel for providing the PM5-2D and MW190-9B strains, Dr. H. Y. Steensma for the *K. lactis rag2::loxP* strain and Dr. E. Boles for the *S. cerevisiae pgi1* mutant and the corresponding wild type strain. We also thank Aida Sánchez López for technical assistance. N.T. is the recipient of an F.P.U. fellowship from the Ministerio de Educación (Spain). This work was funded by grants BMC2003-04992 from MCYT and PGIDT04PXIC10302PN from Xunta de Galicia (Spain).

CHAPTER 7

Cytosolic NADPH concentration modulates the activity of the two external alternative dehydrogenases from *Kluyveromyces lactis* mitochondria

Nuria Tarrío, M. Esperanza Cerdán and M. Isabel González Siso

Submitted for publication

SUMMARY

The mitochondria of the respiratory yeast *Kluyveromyces lactis* are able to reoxidize cytosolic NADPH. We have previously characterized one external alternative dehydrogenase with this activity, Klnde1p, whose sequence is similar to the counterpart enzymes of *Saccharomyces cerevisiae*, although these are NADH-specific. In this work we have characterized the second external alternative dehydrogenase of *K. lactis* mitochondria, Klnde2p. We have studied its role in cytosolic NADPH reoxidation by heterologous expression in *S. cerevisiae* and by construction of *Δklnde1* mutants in both a wild type strain and a *rag2* (phosphoglucose isomerase) mutant that is forced to metabolize the glucose through the pentose phosphate pathway. Although the sequence of Klnde2p is similar to the external calcium-dependent alternative dehydrogenase of *Neuropora crassa*, two differences are that Klnde2p uses NADH or NADPH, and that its activity in isolated mitochondria is not regulated by calcium. The *Δklnde1rag2* double mutant is unable to grow in glucose, which is due not to a down-regulation of Klnde2p in glucose but to the lower affinity for NADPH of Klnde2p *versus* Klnde1p.

INTRODUCTION

The mitochondrion is the cellular organelle responsible for energy production. The electrons produced by oxidation of NADH or NADPH are transported through the mitochondrial respiratory chain to oxygen, generating a proton gradient that leads to ATP synthesis during the oxidative phosphorylation process.

In contrast to mammals, in the inner mitochondrial membrane found in plants and fungi there are alternative dehydrogenases able to oxidize cytosolic and matrix pyridine-nucleotide coenzymes (Dawson, 1979). As opposed to complex I, alternative dehydrogenases are non-proton-pumping and are single polypeptide enzymes instead of polymeric forms. These enzymes, that transfer the electrons to ubiquinone, are external (catalytic site faces towards intermembrane space) or internal (catalytic site faces towards mitochondrial matrix) (Kerscher, 2000; Josep-Horne *et al.*, 2001).

The precise physiological role of alternative dehydrogenases is still unclear; their main function is probably related to the regulation of intracellular redox balance and energy production. We have previously proposed that the characteristics of the mitochondrial alternative NAD(P)H dehydrogenases are related to the type of respiro-fermentative metabolism which predominates in different yeasts (Tarrio *et al.*, 2005, Tarrio *et al.*, 2006).

Kluyveromyces lactis is a respiratory yeast showing a higher use of the pentose phosphate pathway to metabolize the glucose than the fermentative *Saccharomyces cerevisiae* (Jacoby *et al.*, 2003, Blank *et al.*, 2005).

In *K. lactis* mitochondria, where the absence of complex I is known (Josep-Horne *et al.* 2001), we have previously characterized two alternative dehydrogenases whose sequences are similar to those of *S. cerevisiae*, the single internal (Klndi1p) and one external (Klnde1p) (Tarrío *et al.*, 2005). Different from the external enzymes in *S. cerevisiae* (De Risi *et al.* 1997, Luttik *et al.*, 1998; Small and McAlister-Henn, 1998), Klnde1p are able to oxidize NADPH and it is not transcriptionally down-regulated in high glucose concentrations. These characteristics sustain the high activity of the pentose phosphate pathway and respiratory metabolism typical of *K. lactis* (Tarrío *et al.*, 2005).

Up until the present work, Klnde1p is reported as the single external alternative dehydrogenase from yeasts with capacity to oxidize NADPH. Other external alternative dehydrogenases using NADPH have been reported in the filamentous fungus *Neurospora crassa* and in plants (Melo *et al.*, 2001; Carneiro *et al.* 2003; Michalecka *et al.*, 2004).

When the complete sequence of the *K. lactis* genome was available (Dujon *et al.* 2004) we found other putative external alternative dehydrogenase (Klnde2p) showing greater similarity to the external calcium-dependent enzyme from *N. crassa* than to the enzymes from *S. cerevisiae* (Tarrío *et al.* 2006). In this work, we have characterized this sequence and studied its role in the reoxidation of cytosolic NADPH. We have proved that Klnde2p oxidizes NADPH but with lower affinity than Klnde1p.

MATHERIALS AND METHODS

Cloning of the KlNDE2 gene

The DNA and protein sequences were obtained from the *Génolevoures* database (http://cbi.labri.fr/Genolevures/elt/KLLA). The *KlNDE2* coding sequence (ORF from Klla0A:733200...735302 sense p), flanked by 1017 bp upstream and 933 bp downstream, was cloned in the plasmid YEplac195, between the XbaI and SalI restriction sites, using GAP-repair by recombination in *S. cerevisiae*. The resulting construction was named pKlNDE2. The cloned sequence was amplified by PCR from genomic DNA of the *K. lactis* wild type strain CBS2359 using primers that contained 30 nucleotides at the 5´end homologous to the YEplac195 multiple cloning site and 20 nucleotides at the 3´end homologous to the *KlNDE2* sequence.

Sequence analysis

The similarity of the protein sequences was analyzed using BLAST from *Génolevures*. Multiple alignments were carried out using CLUSTAL W (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html). Prediction of mitochondrial targeting sequences was carried out using MitoProt II and prediction of calcium binding domains was carried out using MotifScan, both programs available at ExPASy (http://www.us.expasy.org).

Strains of yeasts

The following *K. lactis* strains were used: the wild type CBS2359 (*MATa*, ATCC8585, NRRL-Y1140), the wild type PM5-3C *(MATa uraA Rag*$^+$) (Wésolowski-Louvel *et al.*, 1992) and the *rag2* (phosphoglucose isomerase) mutant CBS2359 *rag2*::loxP (Steensma and Ter Linde, 2001). The following *S. cerevisiae* strain, supplied by Euroscarf (http://www.uni-frankfurt.de/fb15/mikro/euroscarf/col_index.html), was used: Y00726 (*MATa his3-1 leu2-0 met15-0 ura3 YMR145c::kanMX4*). The ORF YMR145c corresponds to the *NDE1* gene. The *S. cerevisiae* strains ENY.WA-1B (*MATa ura3-52 leu2-3,112 trp1-289 MAL2-8*^{c} *MAL3 SUC3*), the phosphoglucose isomerase mutant EBY22 (ENY.WA-1B *pgi1Δ::TRP1*) (Boles *et al.* 1993) and GH1 (*MATα, trp1-289, leu2-3, leu2-112, gal1Δ152, lys2*) were also used.

Media and culture conditions

Growth and handling of yeasts were carried out according to standard procedures (Kaiser *et al.* 1994). The yeast cells were cultured, unless otherwise stated, in Erlenmeyer flasks at 30°C and 150-250 rpm in the synthetic complete medium CM (Zitomer and Hall 1976) or the dropout media CM-ura (without uracil) and CM-leu (without leucin), containing different carbon sources. YPD (2% glucose, 2% peptone and 1% yeast extract) was also used. The flasks were filled with 40% volume of culture medium. Solid growth media also contained 1.5% agar.

Construction of a S. cerevisiae Δnde1nde2 double mutant

Deletion in the open reading frame YDL085w (*NDE2*) was carried out in the *Δnde1* mutant Y00726 by the short flanking homology (SFH) method described by Wach *et al.* (1994). The *S. cerevisiae NDE2* gene (nucleotides +3 to +1635) was replaced by the *LEU2* gene. The SFH deletion cassette was made with primers homologous to both the *LEU2* gene (20 nucleotides at the 3´end) and the *NDE2* gene (30 nucleotides at the 5' end), using Ylplac128 as template. The Y00726 strain was transformed with the SFH-PCR product. CM-leu was used as culture medium for the selection of transformants. Correct gene deletion was verified by digestion of genomic DNA with *Eco*RV restriction enzyme followed by Southern blotting and hybridization according to the protocol described in the *Digoxygenin labeling and detection kit* (*Roche*). The DNA probe was obtained by PCR using *S. cerevisiae* GH1 genomic DNA as template and specific primers which amplified the region of 560bp downstream of the stop codon of the *NDE2* gene.

KlNDE1 gene deletion in K. lactis wild type and rag2 mutant strains

Deletion in *KlNDE1* gene (nucleotides +3 to +1638) was obtained by the one step method (Rothstein 1991), which was adapted to the low recombination frequency of *K. lactis* by using regions homologous to the target gene greater than 600 bp length. The template of the deletion cassette was made by the procedure described in Zaragoza (2003), and consisted of the kanamycin resistance gene (the *kanMX4* module) flanked by 1088bp homologous to the *KlNDE1* promoter region and 656bp homologous to the *KlNDE1* terminator, in the plasmid YEplac195. The deletion cassette (3.3 Kb) was PCR amplified and the product transformed to the *K. lactis* wild type strain PM5-3C and to the CBS2359 *rag2*::loxP mutant. For selection of transformants, geneticin was added at a final concentration of 0.3 mg/mL to YPD medium. In the wild type strain, correct gene deletion was verified by digestion of genomic DNA with *Eco*RI restriction enzyme followed by Southern blotting and hybridization according to the protocol described in the *Digoxygenin labeling and detection kit* (*Roche*). The DNA probe was obtained by PCR using *K. lactis* CBS2359 genomic DNA as template and specific primers which amplified the region of 560bp upstream of the start codon of the *KlNDE1* gene. In the *rag2* mutant, correct gene deletion was verified by analytical PCR with isolated genomic DNA as template and several combinations of inward primers binding outside the deletion cassette of 3.3 Kb and outward primers binding within the *kanMX4* module.

Yeast transformation

The lithium acetate procedure (Ito *et al.*, 1983) was used.

Isolation of mitochondria

The method described by Herrmann *et al.* was used. Cells were aerobically grown in CM or CM-ura (transformants) with 1% glucose or 1% lactate supplemented with 0.09% glucose, as carbon sources.

Oxygen uptake studies with mitochondrial preparations

Substrate-dependent oxygen consumption rates of isolated mitochondria were determined at 30°C using a Clark-type oxygen electrode (Hansatech) as described by Luttik *et al.* Measurements were made in the absence or presence of 0.25mM ADP, and all respiration was found to be sensitive to antimycin A. Except otherwise stated, the assays were performed at pH 7; respiratory substrates were 0.2mM NADH and 1 mM NADPH; in the experiments to study the effect of calcium, it was added to a final concentration of 0.2µg/mL.

Protein determination

Protein concentration was measured by the method of Bradford (BioRad reagent) using bovine serum albumin as a standard.

Other procedures

Standard procedures for manipulation of nucleic acids were essentially those of Sambrook *et al.* (1989). *Escherichia coli* DH-10B was used for plasmid amplification.

RESULTS

Analysis of the sequence coded by the *KlNDE2* gene

The *KlNDE2* gene was identified in the *K. lactis* complete genome sequence available from the *Génolevoures* database, using BLAST to look for sequences with similarity to Klnde1p (Tarrio *et al.* 2005). *KlNDE2* codes for a putative protein, Klnde2p (KLLA0A08316g), showing 39% identity with Klnde1p, 48% identity with the external calcium-dependent NADPH dehydrogenase from *N. crassa* (Melo *et al.* 2001) and lower identity (24 and 25%, respectively) with *S. cerevisiae* Nde1p and Nde2p (Luttik *et al.* 1998; Small and McAlister-Henn, 1998).

As predicted by the program MitoProt II (Claros and Vicens, 1996) with a probability of 0.96, Klnde2p has an N-terminal mitochondrial-targeting sequence of 59 amino acids (Fig. 1). Klnde2p and also Klnde1p have predicted mitochondrial-targeting sequences longer than Klndi1p (25 amino acids) (Tarrío et al 2005). Similarly, in *S. cerevisiae* the external alternative dehydrogenases have predicted mitochondrial-targeting sequences longer than the internal enzyme (Luttik *et al.*, 1998).

*MFATGISKGLIGVALPIRSIHGSMRGPIQTVNKLPLRAVCLASFKPSVRNFHVSSCLRS*EIEQEKSTPPV
GLVKTSGFTSKLGKLVKWSIFTAGIVASGVGAIIIGFFIYDSTTYKSCGTAVEIKVPKLALNPELGGPEN
LPILRETLDAYDSEMKELTSYKPKLVVLGSGWASVGLLLKNLNPGDYDVTVVSPQNYFLFTPLLPSAATGT
LEVKSLMASIRKIVNDVNGHYLEAYAEKVEFDEKLIKVSQINTKTGAKDSFYLPYDKLVIAVGSTSNTHG
VEGLQYCSRLKTAEDAITLRKKIKTLLERACLPTTSDEERRRLLSFVVCGGGPTGVEFAAAEVFDLLNEDL
PSMYPRILRQQLSVHVIQSRSNILNTYDEKISEYATQRFRKETIDVLTNSRVERILPDRVIFKQKDDKTG
EVELKEIPFGICLWSTGVSQNPLTKQVVHSLAHSQRNKRAIETDSYLRVIGAPTEDVYAIGDCSTVRTDL
ADHTADYIRRFIVNRHLSLTRSNEIITDEDIKHLSLSYNEIIDIAKQVARRHPQTREHLIHLEDDLPKYD
VNKSGQLNFDQISTLLREVETKVTSLPATAQRAHQQGKYLGKKLTKVARSANKDSIQGIHEKGIDEKVYR
PFRYVHLGSLAYIGNSAVFDLPGYSFVGGLIAMYLWRSIYFAQTVSWRTRVLLFMDWLKRGMFGRDILSE

Figure 1: Predicted amino acid sequence of Klnde2p. Glycine-rich motifs are underlined, the putative missed acid charge is squared and the putative EF-hand motif is double-line squared.

Fig. 1 shows that Klnde2p contains two conserved glycine rich motifs (GXGXXG) for binding the substrate (NADH or NADPH) and the FAD cofactor (Lesk, 2001). The first of these fits into the criteria for NADPH binding: the last of the three highly conserved glycine residues is replaced by serine, and a negatively charged aminoacid downstream, which is conserved in the NADH-binding proteins, is replaced by an asparagine, thus avoiding its unfavourable interaction with the also negatively charged 2´-phosphate of NADPH, as previously proposed (Lesk, 2001). Moreover, Klnde2p contains a calcium binding motif predicted by the Motif Scan program as being questionable (Fig. 1). The presence of a calcium binding motif and the loss of a conserved negative charge downstream of the first glycine rich motif, are the most characteristic differences between the second and the first external alternative dehydrogenases from *K. lactis* (Fig. 1, Tarrío *et al.*, 2005).

These sequence-based functional predictions have been tested experimentally as described in the following sections.

KlNDE2 codes for an external mitochondrial dehydrogenase that uses NADPH and NADH as substrates

The features above exposed of the Klnde2p sequence suggest its ability to use NADPH. We tested this hypothesis, in the first place, by heterologous expression in *S. cerevisiae*. We built a *S. cerevisiae* double mutant in the external alternative dehydrogenases by deletion of the *NDE2* gene in the *Δnde1* mutant, as described in Materials and Methods. We verified that the isolated mitochondria of this mutant were unable to oxidize both NADH and NADPH (Table I). Then we transformed the *S. cerevisiae Δnde1nde2* strain with the plasmid pKlNDE2, described in Materials and Methods, and also with the plasmid pKlNDE1, expressing the *KlNDE1* gene from its own promoter as described in Tarrío *et al.*, 2005. The isolated mitocondria of the transformants expressing either *KlNDE1* or *KlNDE2* acquire the capacity to oxidize both NADH and NADPH, with the same efficiency (Table 1). These results also confirm that the previously reported (Tarrío et al 2005) increase in NADH-oxidation rate by isolated mitochondria of the *S. cerevisiae nde1* single mutant when expressing the *KlNDE1* gene, was actually due to the activity of Klnde1p and not to an up-regulation of the *S. cerevisiae* Nde2p, since the increase of activity is of the same magnitude in the single *nde1* (Tarrío et al 2005) and double *nde1nde2* (this work) mutants.

Table I. Substrate-dependent rates of oxygen consumption by isolated mitochondria from the *S. cerevisiae Δnde1nde2* mutant, the *Δnde1nde2* mutant transformed with an episomic plasmid expressing the *KlNDE1* gene and the *Δnde1nde2* mutant transformed with the same episomic plasmid expressing the *KlNDE2* gene.

STRAIN		**NADH**	**NADPH**
Δnde1nde2	Oxygen uptake rate	N.D.	N.D.
	Respiratory control	N.D.	N.D.
Δnde1nde2 + pKlNDE1	Oxygen uptake rate	0.033 ± 0.01	0.034 ± 0.01
	Respiratory control	1.72 ± 0.13	1.47 ± 0.22
Δnde1nde2 + pKlNDE2	Oxygen uptake rate	0.036 ± 0.01	0.032 ± 0.01
	Respiratory control	1.26 ± 0.19	1.40 ± 0.30

Cells were grown in aerobic lactate cultures as described in Herrmann *et al.* (2001). The oxygen uptake rates ($\mu molO_2/min*mg$ protein) were measured in the presence of 0.25mM ADP; respiratory control values represent the ratio between respiration rates in the presence and absence of ADP. Experimental results are the mean ± S.D. of $4 \leq n \leq 8$ (at least two analytical measurements with mitochondria isolated from two independent cultures of the untransformed *Δnde1nde2* mutant and three independent cultures of three different transformants). N.D. = not detected.

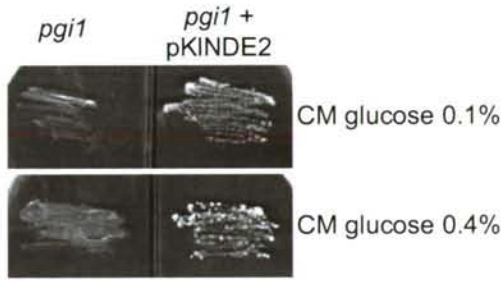

Figure 2: *KlNDE2* overexpression restores the growth in glucose of *S. cerevisiae pgi1* mutant. The *pgi1* mutant (EBY22) transformed with an episomic plasmid (YEplac195) carrying the *KlNDE2* gene grown in CM with 0.1% and 0.4% glucose for 20 days at 30ºC.

To obtain more evidence supporting the ability of Klnde2p to reoxidize the pentose phosphate pathway-NADPH, the plasmid pKlNDE2 was introduced into the *S. cerevisiae pgi1* mutant EBY22, which is unable to grow in media with 0.1% glucose or higher concentrations, due to a depletion of $NADP^+$ that blocks the pentose phosphate pathway (Boles et al 1993). We have previously demonstrated that the overexpression of the *KlNDE1* gene restored the growth of the *pgi1* mutant on media with 0.1, 0.5 and 2% glucose, because Klnde1p reoxidizes the NADPH produced in the pentose phosphate pathway (Tarrio *et al.* 2005, Tarrio *et al.*, 2006). The transformants with the plasmid expressing the *KlNDE2* gene under its own promoter region were able to grow

on CM-ura with glucose concentrations from 0.1 to 0.4%, but not 0.5% or higher (Fig. 2). This result suggests that Klnde2p is an external dehydrogenase able to oxidize cytosolic NADPH and also that Klnde2p is less efficient in pentose phosphate pathway-NADPH reoxidation than Klnde1p, at least in physiological conditions in the *S. cerevisiae pgi1* mutant.

Deletion of the *KlNDE1* gene in a *K. lactis* wild type strain

To investigate the specific role of Klnde1p and Klnde2p in *K. lactis* mitochondria, we deleted the *KlNDE1* gene in the chromosomal DNA from a *K. lactis* wild type strain (PM5-3C), as described in Materials and Methods. The mitochondria of the *Δklnde1* strain contain Klnde2p as the single external alternative dehydrogenase.

To investigate the phenotype of the *Δklnde1* mutant, we compared its growth rate with the growth rate of the *K. lactis* wild type strain in shake-flask cultures with different carbon sources (Table II). There are no significant differences in the growth on different carbon sources, neither fermentable nor non-fermentable, between the *K. lactis* strains wild type and *Δklnde1*.

Table II. Duplication time (h) of *Kluyveromyces lactis* PM5-3C and isogenic *Klnde1* null mutant in CM with different carbon sources.

Carbon source	**Duplication time (h)**	
	PM5-3C	***Δnde1***
Glucose (2%)	3.2 ± 0.6	3.9 ± 0.4
Fructose (2%)	3.2 ± 0.4	3.2 ± 0.4
Galactose (2%)	3.2 ± 0.7	3.8 ± 0.3
Glycerol (5%)	3.6 ± 0.4	4.0 ± 0.1
Lactate (2%)	15.6 ± 1.7	10.1 ± 1.4
Lactate (2%) + glucose (0.05%)	6.1 ± 0.1	6.0 ± 0.1
Ethanol (5%) + glucose (0.05%)	4.3 ± 0.1	4.3 ± 0.1

Data are mean ± S.D. from two independent shake-flask experiments.

We also measured the substrate-dependent rates of oxygen consumption by isolated mitochondria from the *Δklnde1* null mutant and the corresponding *K. lactis* wild type strain growing in lactate media. The ratios in NADH or NADPH oxidation by the *Δklnde1* mutant isolated mitochondria in comparison with the wild type isolated mitochondria did not decrease (Table III), the rate of NADPH oxidation was even slightly higher in the mutant.

Table III. Substrate-dependent rates of oxygen consumption by isolated mitochondria from *K. lactis* wild type strain (PM5-3C) and *Δklnde1* mutant grown in aerobic 1% lactate media.

STRAIN		NADH	NADPH
PM5-3C	Oxygen uptake rate	0.08 ± 0.01	0.08 ± 0.02
	Respiratory control	1.45 ± 0.06	2.34 ± 0.86
Δklnde1	Oxygen uptake rate	0.11 ± 0.01	0.16 ± 0.00
	Respiratory control	1.93 ± 0.84	1.45 ± 0.31

The oxygen uptake rates ($\mu molO_2/min*mg$ protein) were measured in the presence of 0.25mM ADP; respiratory control values represent the ratio between respiration rates in the presence and absence of ADP. Experimental results are the mean ± S.D. of $5 \leq n \leq 7$ (at least two analytical measurements with mitochondria isolated from two independent cultures of each strain).

The deletion of *KlNDE1* in a *K. lactis* wild type strain showed, therefore, no obvious phenotype, as reported for *S. cerevisiae NDE2* (Luttik *et al.* 1998). However, the result exposed in the previous section from the heterologous expression of Klnde2p in the *S. cerevisiae pgi1* mutant suggested a more important role of Klnde1p than Klnde2p in reoxidation of cytosolic NADPH. We next investigated this.

Deletion of the *KlNDE1* gene in a *K. lactis rag2* mutant

The *K. lactis* CBS2359 *rag2*::loxP strain which is devoid of phosphoglucose isomerase, can grow in media with glucose as sole carbon source (Steensma and Ter Linde, 2001) in contrast to the *S. cerevisiae pgi1* mutant (Boles *et al.*, 1993). We previously proved that the existence in *K. lactis* of at least one external alternative dehydrogenase using the NADPH from the pentose phosphate pathway was responsible for this metabolic difference (González Siso *et al.*, 1996a; Tarrío *et al.* 2005). We have demonstrated in this work that a second alternative external dehydrogenase of *K. lactis* mitochondria is also able to use NADPH. Then, to evaluate the relative importance of both enzymes, Klnde1p and Klnde2p, in the reoxidation of the NADPH produced in the pentose phosphate pathway in *K. lactis*, we deleted the *KlNDE1* gene in the *rag2* mutant as described in Materials and Methods.

The *K. lactis Δklnde1rag2* double mutant obtained lost the ability to grow in the two carbon sources where the *rag2* single mutant grows well: glucose and galactose. This double mutant grew very poorly in YP medium without added carbon source and only during a few generations. This result shows that Klnde1p is essential for the

growth of the *rag2* mutant in glucose and suggests that this protein has the major capacity of cytoplasmic NADPH reoxidation in the cells of *K. lactis*.

This conclusion was in agreement with the results of the heterologous expression of *KlNDE1* and *KlNDE2* in the *S. cerevisiae pgi1* mutant but in contrast with the NADPH oxidation rates obtained with isolated mitochondria of the *S. cerevisiae nde1nde2* mutant expressing the *KlNDE1* gene compared to *KlNDE2*, and with isolated mitochondria of the *K. lactis nde1* mutant compared to the wild type. The next question to investigate was, therefore, why in the experiments with isolated mitochondria Klnde2p seems to be equally or more efficient in NADPH oxidation while in experiments *in vivo* Klnde1p seems to be more efficient? Four factors that could possibly modulate the activity of both enzymes *in vivo* were envisaged: pH, glucose down-regulation of *KlNDE2*, calcium, and cytosolic NADPH levels.

The effect of pH on Klnde1p and Klnde2p activities

Since we had made all the measurements with isolated mitochondria at pH 7 and the activity of the two *N. crassa* external alternative dehydrogenases is distributed in different pH ranges (Melo *et al.*, 2001; Carneiro *et al.*, 2003), to understand why the *rag2* mutant lose the ability to grow in glucose when the *KlNDE1* gene is deleted but *KlNDE2* remains, we measured the NADH and NADPH oxygen uptake rates by isolated mitochondria from the *K. lactis* wild type and the *Δklnde1* mutant strains at different pH values. The results (Fig. 3) show that the effect of pH on external dehydrogenase activity is similar in mitochondria from both strains.

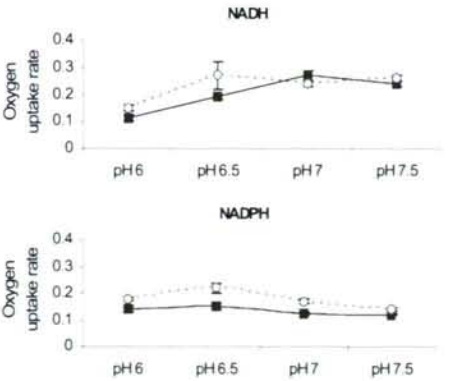

Figure 3: Exogenous NADH and NADPH oxidation at pH 6.0-7.5 by isolated mitochondria from *K. lactis* wild type (PM5-3C) and *Δnde1* mutant. Black squares correspond to the *K. lactis* wild type and white circles to the *Δnde1* mutant. The oxygen uptake rates ($\mu molO_2/min*mg$ protein) were measured in the presence of 0.25mM ADP. Respiratory control values, the ratio between respiration rates in the presence and absence of ADP, are in the wild type of 1.35 ± 0.16 for NADH and of 1.16 ± 0.13 for NADPH, being in the *Δnde1* mutant of 1.25 ± 0.15 for NADH and of 1.16 ± 0.14 for NADPH. Cells were grown in aerobic 1% glucose media. Experimental results are the mean ± S.D. of $2 \leq n \leq 4$.

Klnde2p is not down-regulated when *K. lactis* grows in glucose

To test if a down-regulation of Klnde2p in glucose prevented the growth in this carbon source of the *Δklnde1rag2* mutant, we measured at the optimum pH the NADH and NADPH oxygen uptake rates by isolated mitochondria from the *K. lactis* wild type and the *Δklnde1* mutant growing in 1% glucose media and compared the values obtained (Table IV) with those obtained with mitochondria from lactate media (Table III).

Table IV. Substrate-dependent rates of oxygen consumption by isolated mitochondria from *K. lactis* wild type strain (PM5-3C) and *Δklnde1* mutant grown in aerobic 1% glucose media.

pH	STRAIN		NADH	NADPH
6.5	PM5-3C	Oxygen uptake rate	0.20 ± 0.01	0.17 ± 0.02
		Respiratory control	1.33 ± 0.10	1.23 ± 0.13
	Δklnde1	Oxygen uptake rate	0.27 ± 0.04	0.24 ± 0.03
		Respiratory control	1.38 ± 0.13	1.36 ± 0.19
7	PM5-3C	Oxygen uptake rate	0.26 ± 0.02	0.17 ± 0.03
		Respiratory control	1.43 ± 0.13	1.34 ± 0.20
	Δklnde1	Oxygen uptake rate	0.20 ± 0.03	0.15 ± 0.02
		Respiratory control	1.54 ± 0.35	1.39 ± 0.17

The oxygen uptake rates ($\mu molO_2/min*mg$ protein) were measured in the presence of 0.25mM ADP; respiratory control values represent the ratio between respiration rates in the presence and absence of ADP. The measurements were performed at pH 6.5 and pH 7. Experimental results are the mean ± S.D. of $5 \leq n \leq 7$ (at least two analytical measurements with mitochondria isolated from two independent cultures of each strain).

As with mitochondria from lactate cultures (Table III), the rate of NADH or NADPH oxidation was not lower with *Δklnde1 versus* wild type mitochondria coming from glucose cultures (Table IV).

The comparison of results in Tables III and IV also show that down-regulation by glucose does not affect *KlNDE2* expression, because the NADH and NADPH oxidation rates are not lower with mitochondria coming from glucose *versus* lactate cultures. The NADPH oxidation rates by isolated mitochondria are the same when *Δklnde1* mutant is cultured in glucose or lactate, being the NADH oxidation rates when

the *Δklnde1* mutant is grown in glucose 1.8 times higher compared to lactate media. Concerning wild type, NADH and NADPH oxidation rates by isolated mitochondria when the wild type is cultured in glucose are, respectively, 3.3 or 2.1 times higher compared to lactate media. The ratios of activity glucose/lactate obtained in the wild type *versus* the *Δklnde1* mutant suggest that there is an up-regulation in glucose which operates mainly on Klnde1p. However, the kind of regulation which operates is still uncertain. It has been described that *KlNDE1* mRNA levels in high glucose were similar to those obtained in low glucose or in lactate media (Tarrio *et al.*, 2005), therefore the transcriptional regulation cannot be involved.

Klnde2p activity is not calcium-dependent

Klnde2p, different to Klnde1p, contains a calcium binding (E-F hand) motif as predicted by the Motif Scan program (Fig. 1). In plants and *N. crassa* there are alternative dehydrogenases containing an E-F hand motif that are calcium-dependent (Michalecka *et al.* 2004; Melo *et al.* 2001). Then, we tested if calcium regulates the Klnde2p activity.

First, we used the *S. cerevisiae Δnde1nde2* strain transformed with the plasmid expressing the *KlNDE2* gene and measured the NADH and NADPH oxidation by isolated mitochondria before and after addition of calcium (Fig. 4A). We made the assays at pH 7-7.5 because it has been demonstrated that the calcium-dependent Nde1p from *N. crassa*, which has a similar E-F hand motif, has the highest differences in the activity between presence and absence of calcium at this pH range (Melo *et al.*, 2001). The results show that Klnde2p is not regulated by calcium in *S. cerevisiae* isolated mitochondria. As control, we made the same assay, but only at pH 7, in *S. cerevisiae Δnde1nde2* mutant transformed with the plasmid carrying the *KlNDE1* gene, since Klnde1p does not present E-F hand motif as predicted by Motif Scan program, being the oxygen uptake rates in presence of ADP and calcium 0.032 ± 0.01 for NADH and 0.031 ± 0.01 for NADPH, therefore there are not differences comparing these rates with the rates obtained from this transformant in the absence of calcium, as shown in Table1.

Second, we used the *Δklnde1* mutant that only contains Klnde2p as external alternative dehydrogenase, and measured the effect of calcium on the oxygen uptake rate by isolated mitochondria from cells growing in lactate cultures (Fig. 4B). The result is the same in the *K. lactis* and *S. cerevisiae* isolated mitochondria, there is not effect of calcium on Klnde2p activity. Moreover, the calcium effect has also been studied in isolated mitochondria from the *Δklnde1* mutant growing in glucose and from the wild type strain growing in lactate and glucose, obtaining the same result (data not shown).

Also, a range of calcium concentrations was tested from 0.04 to 0.4 □g/mL (data not shown). Therefore, we can conclude that calcium is not involved in the regulation of the Klnde2p activity of isolated mitochondria.

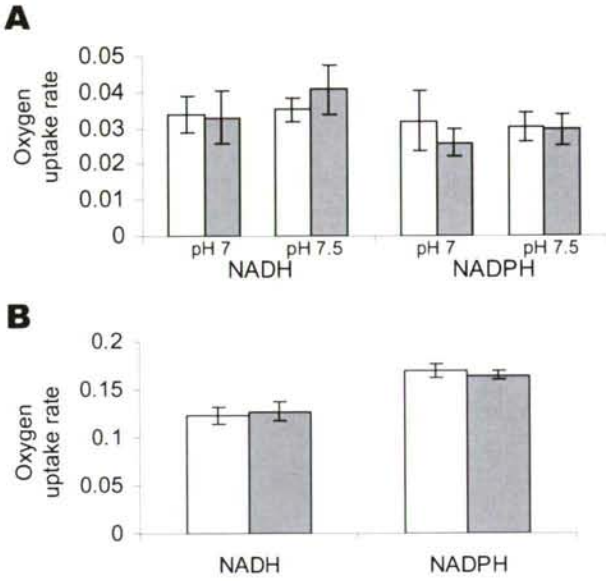

Figure 4: Calcium influence on Klnde2p activity. (A) NADH and NADPH oxidation by isolated mitochondria from aerobic lactate cultures of the *S. cerevisiae Δnde1nde2* mutant transformed with an episomic plasmid expressing the *KlNDE2* gene from *K. lactis*. White bars correspond to absence of calcium and grey bars to presence of calcium. The oxygen uptake rates ($\mu molO_2/min*mg$ protein) were measured in the presence of 0.25mM ADP. Respiratory control values are for NADH in pH 7 of 1.27 ± 0.20 and in pH 7.5 of 1.26 ± 0.30, for NADPH in pH 7 of 1.32 ± 0.24 and in pH 7.5 of 1.37 ± 0.20. Experimental results are the mean ± S.D. of $3 \leq n \leq 7$ (at least two analytical measurements with mitochondria isolated from two independent cultures of two different transformants). (B) NADH and NADPH oxidation by *K. lactis Δnde1* mutant isolated mitochondria. The cells were grown in aerobic lactate media. The assays were performed at pH 7. White bars correspond to absence of calcium and grey bars to presence of calcium. The oxygen uptake rates ($\mu molO_2/min*mg$ protein) were measured in the presence of 0.25mM ADP. Respiratory control values are for NADH 1.09 ± 0.01 and for NADPH 1.22 ± 0.02. Experimental results are the mean ± S.D. of n = 2.

The differences in the physiological role between the two *K. lactis* external alternative dehydrogenases lie in their Michaelis-Menten constants

Mitochondrial external alternative dehydrogenase activity from plants has been reported to be regulated by cytosolic factors such as substrate concentration in addition to calcium (Møller, 2001). Moreover, in *K. lactis*, comparing the *rag2 versus* the wild type strain in glucose, both a higher external dehydrogenase activity in isolated mitochondria and an increased *KlNDE1* transcription have been reported (Overkamp *et al.* 2002, Tarrio et al 2005). In the *rag2* mutant in glucose, there is higher use of the pentose phosphate pathway and production of higher levels of cytosolic NADPH. We

therefore studied the influence of NADPH concentration on the external alternative dehydrogenase activity in isolated mitochondria from wild type and *Δklnde1* strains.

The NADPH oxidation by wild type and *Δklnde1* isolated mitochondria was assayed using different concentrations of NADPH from 0.016 to 1mM. The data conform with a Michaelis-Menten Kinetics. We calculated the K_M(NADPH) and V_{max} from the double-reciprocal and Hanes–Woolf representations with two independent mitochondria isolations (Fig. 5), the wild type isolated mitochondria being K_M(NADPH) = 0.05 ± 0.02mM and V_{max} = 0.16 ± 0.04 oxygen uptake rate and for *Δklnde1* isolated mitochondria K_M(NADPH) = 0.11 ± 0.03mM and V_{max} = 0.17 ± 0.02 oxygen uptake rate. The K_M is 2-fold higher in *Δklnde1* than in wild type isolated mitochondria. This fact suggests a lower affinity for NADPH of Klnde2p than Klnde1p. Therefore, the cytosolic NADPH concentration may determine the relative activity of Klnde1p and Klnde2p.

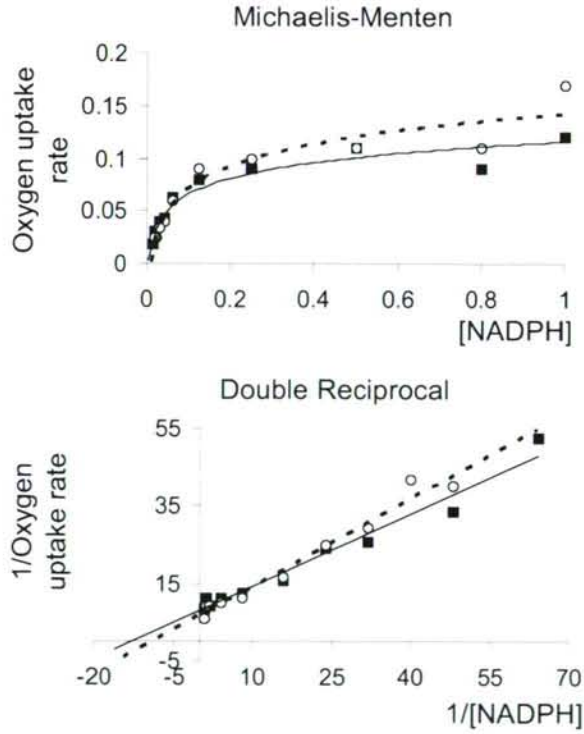

Figure 5: Michaelis-Menten parameters for *K. lactis* wild type (PM5-3C) and *Δnde1* mutant external NADPH dehydrogenase activity of isolated mitochondria. Direct plot of the data from an independent mitochondria isolation in the Michaelis-Menten and Double Reciprocal graphics (r = 0.97 ± 0.01). The oxygen uptake rates ($\mu molO_2$/min*mg protein) were measured in the presence of 0.25mM ADP. Black squares correspond to the *K. lactis* wild type and white circles to the *Δnde1* mutant. The cells were grown in aerobic 1% glucose media.

We also measured the NADH oxidation rate by isolated mitochondria from the *K. lactis* wild type and *Δnde1* mutant with different NADH concentrations from 0.008 to 0.2mM (data not shown) but, for technical reasons, it was impossible to adjust the data to a Michaelis-Menten Kinetics. This may be related to the physiological NADH concentration in the cytoplasm which in plants was estimated at around 0.001mM (Heineke, *et al.*, 1991; Møller, 2001). The relative importance of Klnde1p and Klnde2p in reoxidation of cytosolic NADH will be the subject of further investigation.

DISCUSSION

We have previously characterized in *K. lactis* two mitochondrial alternative dehydrogenases, the internal that replaces complex I, and one external that is able to oxidize cytoplasmic NADH and NADPH (Tarrio *et al.*, 2005). In this work we have characterized another external alternative dehydrogenase from *K. lactis* mitochondria, Klnde2p. The study about the physiological role of these enzymes is necessary for understanding the diverse patterns of respiro-fermentative metabolism in yeasts. We have proposed that the metabolic differences between the fermentative *S. cerevisiae* and the respiratory *K. lactis* are more dependent on the characteristics of the external alternative dehydrogenases than on the internal enzymes, the major differences being the presence of down-regulation in glucose growing cells and the incapacity to oxidize NADPH of *S. cerevisiae* external alternative dehydrogenases (De Risi et al 1997, Luttik et al 1998, Tarrio *et al.*, 2005). The data presented in this work reveal that the second external alternative dehydrogenase from *K. lactis* can also oxidize NADH and NADPH and that growth of the yeast in glucose *versus* lactate does not decreases this enzyme activity in isolated mitochondria.

On the other hand, in contrast with *S. cerevisiae NDE1* gene and *N. crassa NDE1* and *NDE2* genes (Luttik *et al.* 1998; Small and McAlister-Henn 1998, Melo *et al.* 2001, Carneiro et al 2003), when *KlNDE1* gene is deleted in a *K. lactis* wild type strain, we did not find lower NADH or NADPH oxidation rates by the mutant than by the wild type isolated mitochondria. This fact, which was not pH-dependent, suggested that *K. lactis* can modulate the synthesis or transport of the two external alternative dehydrogenases in relation to cell necessities. However, two results suggested that, in the cell, the Klnde1p and Klnde2p activities do not have exactly the same role. One of them was that the *K. lactis* phosphoglucose isomerase mutant, *rag2*, which metabolizes all glucose through the pentose phosphate pathway generating a surplus of NADPH, is unable to grow in glucose if the *KlNDE1* gene is deleted, and therefore the Klnde2p activity in physiological conditions is not high enough to allow the growth in

glucose of this mutant. The second one was that the *S. cerevisiae* phosphoglucose isomerase mutant, *pgi1*, transformed with an episomic plasmid expressing the *KlNDE2* gene recovered the ability to grow in glucose only until a glucose concentration of 0.4% whereas the same mutant transformed with the same episomic plasmid expressing the *KlNDE1* gene recovered the ability to grow in media with glucose concentration of 2% (Tarrio *et al.*, 2005). The explanation for the loss of the *Δklnde1rag2* mutant capacity to grow in glucose, and the differences in the growth in glucose of the *pgi1* mutant transformed with either one of the two *K. lactis* genes is the different efficiency, *in vivo*, in NADPH reoxidation of these proteins, demonstrating a major efficiency in Klnde1p.

As a consequence, once glucose down-regulation of Klnde2p is excluded, we attributed to some cytosolic parameter the responsibility for the differences in the activity of the two *K. lactis* external enzymes, and we focused on the identification of this parameter. Møller (2001) reported that the presence of calcium and substrate concentration may modulate the activity of the external alternative dehydrogenases in plants. We studied the influence of these two factors in the activity of the two *K. lactis* external alternative dehydrogenases and the results suggested that substrate concentration is involved in the modulation of activities, but not calcium presence in spite of the fact that Klnde2p contains a putative calcium binding domain. The K_M(NADPH) of Klnde2p is higher (approximately double) than that of Klnde1p; therefore, the NADPH concentration in the cytoplasm of the *S. cerevisiae pgi1* or *K. lactis rag2* mutants may determine that Klnde1p is operating at its maximum activity whereas Klnde2p is not, the Klnde2p activity alone not being high enough to reoxidize the NADPH produced by the pentose phosphate pathway when the glycolysis is interrupted in the phosphoglucose isomerase step.

Another parameter that can contribute to the loss of the ability to grow in glucose of the *Δklnde1rag2* mutant is the demonstrated up-regulation of Klnde1p activity in isolated mitochondria from cells grown in glucose cultures.

The data presented in this work support that the characteristics of the mitochondrial external alternative dehydrogenases reflect an adaptation to the life-style of the organisms. Thus, *K. lactis*, as a model of facultative respiratory yeast, needs external alternative dehydrogenases with capacity to oxidize NADPH and not down-regulated by high glucose concentrations, to metabolize the glucose preferentially through the pentose phosphate pathway and in consequence by respiration.

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CHAPTER 8

Subcellular localization of *Kluyveromyces lactis* glutathione and thioredoxin reductases

A part of this work was carried out in Department of Cell and Molecular Biology, Lundberg Laboratory at University of Göteborg, Sweden

INTRODUCTION

Saccharomyces cerevisiae is a degenerate tetraploid resulting from whole-genome duplication that occurred during evolution after the divergence of *Saccharomyces* from *Kluyveromyces*. As a consequence, many of the genes that were retained in duplicate in *S. cerevisiae* are present in a single copy in *K. lactis* (Wolfe and Shields, 1997). Examples are found in the group of genes from the oxidative stress response that are hitherto mostly uncharacterized in *K. lactis*. This yeast has been proposed as a model to study the antioxidant defence mechanisms, alternative to *S. cerevisiae*, because the metabolism of *K. lactis* is more respiratory and as a result higher production of reactive oxygen species by the mitochondria is expected (Tarrio *et al.* 2004)

In eukaryotes, the oxidative stress defence proteins often have mitochondrial and cytosolic isoforms that may either be encoded by different genes or respond to dual targeting of a single gene product. Thus, in *S. cerevisiae* there are two genes encoding thioredoxin reductases, one of them (*TRR1*) codes for the cytosolic isoform and the other (*TRR2*) codes for the mitochondrial isoform (Grant, 2001), whereas the single *S. cerevisiae GLR1* gene has been reported to code for both cytoplasmic and mitochondrial isoforms of glutathione reductase due to two alternative translation initiation sites (Outten and Culotta, 2004). In the *K. lactis* genome there is a single gene (*KlTRR1*) encoding thioredoxin reductase and a single gene (*KlGLR1*) encoding glutathione reductase (Tarrio *et al.* 2004). As in other organisms, both enzymes might be located in the mitochondria and cytosol. The aim of this work is to determine the intracellular compartmentalization of *K. lactis* glutathione and thioredoxin reductases.

MATERIALS AND METHODS

Strains, media and culture conditions

The following *S. cerevisiae* strains, supplied by Euroscarf (http://www.uni-frankfurt.de/fb15/mikro/euroscarf/col_index.html), were used: BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ*), BY4742 (*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ*), Y12737 (BY4742, *YPL091w::kanMX4*), the ORF YPL091w corresponds to the *GLR1* gene, Y11934 (BY4742, *YHR106w::kanMX4*), the ORF YHR106w corresponds to the *TRR2* gene.

Growth and handling of yeasts were carried out according to standard procedures (Kaiser *et al.* 1994). The yeast cells were cultured, unless otherwise stated, in Erlenmeyer flasks at 30ºC and 150-250 rpm in the synthetic complete medium CM (Zitomer and Hall 1976) or the dropout medium CM-ura (without uracil) containing different carbon sources. The flasks were filled with 40% volume of culture medium. Solid growth media also contained 1.5% agar.

Plasmid constructions and expression of the fusion proteins

The *KlGLR1* and *KlTRR1* genes were inserted in the 2-micron plasmid pYES2-GFP under the control of the GAL1 promoter. The resulting constructions (pKlGLR1-GFP and pKlTRR1-GFP) coded for hybrid proteins with one copy of GFP (green fluorescent protein) fused to the C-terminus of Klglr1p or Kltrr1p. The constructions were made by GAP-repair

through recombination. The genes (from nucleotides -3 to +1452 for *KlGLR1* or to +1047 for *KlTRR1*) were amplified by PCR with primers with 30 flanking nucleotides homologous to the vector.

Yeasts were transformed utilising the method of Ito *et al.* (1983)

To induce fluorescence, the transformed cells were cultured overnight in CM-ura with 2% glucose. The following day the cells were washed, transferred to CM-ura with 2% galactose and incubated during 4 hours. They were then examined under the microscope.

Mitochondria staining

To stain mitochondria, 0.1mL from the culture was carried to a tube and 1μL MitoTracker (Molecular Probes) was added. The mix was incubated to room temperature during 15 minutes. It was then examined under the microscope.

Fluorescence microscopy

Specific filters were used in a Leica microscopy to observe fluorescence due to GFP and MitoTracker. The sample was freshly cultured without any treatment.

Confocal microscopy

To observe GFP the excitation λ was 450-490 nm and the emission λ was 520 nm and to observe MitoTracker the excitation λ was 644 nm and the emission λ was 665nm in a Leica confocal microscopy. The sample was freshly cultured without any treatment.

RESULTS AND DISCUSSION

As predicted by the program Mitoprot II (Claros and Vicens, 1996), *KlGLR1* and *KlTRR1* encoded proteins both have a putative mitochondrial-targeting sequence (p=0.97 and p=0.99, respectively). The *KlGLR1* and *KlTRR1* genes show two in-frame methionine start codons, the second methione is just downstream from the putative cleavage site for mitochondrial processing (Fig.1). Therefore, two alternative translation initiation sites could be operating.

KlGLR1

```
guu aug ucc aua cgu aau aca auc aau aga auc auu auu aga aga aac aug
     M   S   I   R   N   T   I   N   R   I   I   I   R   R   N   M
```

KlTRR1

```
ugu aug cuu uua gug aga aau agu aca uua gga cga uua uca uca uua aga
     M   L   L   V   R   N   S   T   L   G   R   L   S   S   L   R

ggg uuu uuu aga aau auc aac gag agu aau aua uuc uac aga aug
 G   F   F   R   N   I   N   E   S   N   I   F   Y   R   M
```

Figure 1: Predicted mRNA and protein sequences of *K. lactis* glutathione and thioredoxin reductases. The mitochondrial import sequences are underlined and the two methionines are in squares.

To test this hypothesis, the *S. cerevisiae Δglr1* and *Δtrr2* mutants were transformed with the constructions pKlGLR1-GFP and pKlTRR1-GFP, respectively. Fig. 2 shows the fluorescence yielded by galactose-induced expression of these gene

fusions, with and without treatment with 1mM and 2mM tBOOH (tert-butyl hydroperoxide) during 1 hour.

For both proteins studied, KlGlr1p and KlTrr1p, distinct galactose-dependent fluorescence has been observed. In both cases the localization appears in the cytoplasm, the localization in the mitochondria is uncertain because dots close to the plasmatic membrane are observed but they do not look like the typical tubular network of yeast mitochondria. The overall fluorescence is slightly higher under the oxidative stress conditions than without treatment, but it is low in both cases.

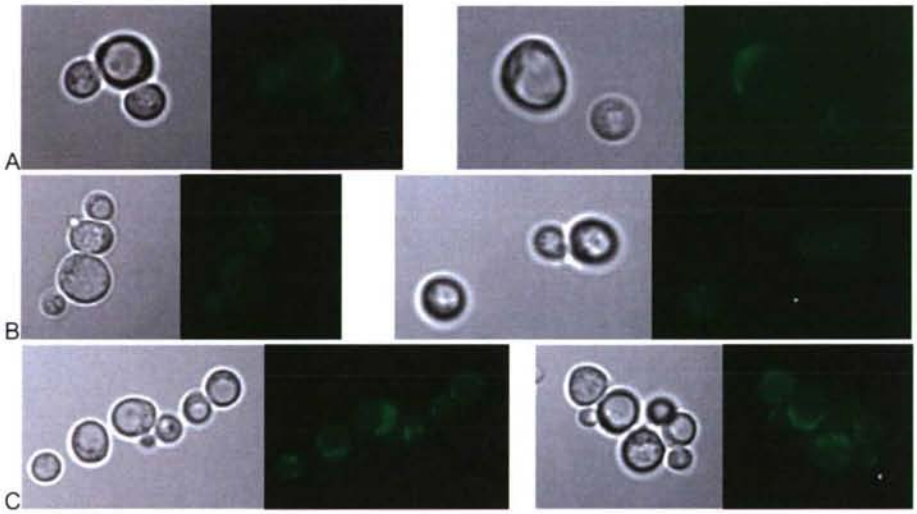

Figure 2: (A) Fluorescence microscopy with GPF filter. pKlGLR1-GFP expression in the *S. cerevisiae Δglr1* mutant (left) and pKlTRR1-GFP expression in the *S. cerevisiae Δtrr1* mutant (right). (B) pKlGLR1-GFP expression in the *S. cerevisiae Δglr1* mutant with 1mM tBOOH treatment (left) and 2mM tBOOH treatment (right). (C) pKlTRR1-GFP expression in the *S. cerevisiae Δtrr1* mutant with 1mM tBOOH treatment (left) or 2mM tBOOH treatment (right).

Then, to study if the overexpression of these proteins was toxic to the cells, we cultivated the transformed cells in glucose and in galactose with different concentrations of tBOOH (Fig. 3). The lack of growth in galactose media, which was corroborated in liquid cultures (data no shown), indicates some toxicity problem.

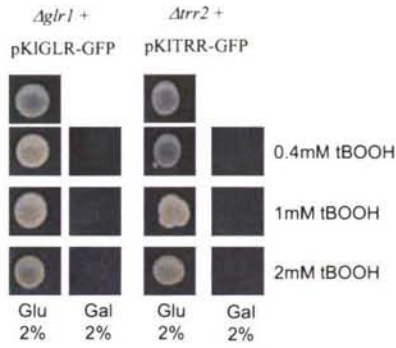

Figure 3: Growth of *Δglr1* and *Δtrr2* mutant expressing the *KIGLR1* and *KITRR1* genes, respectively, after 3 days on glucose (glu) or galactose (gal). The initial OD_{600} is 1 and the serial dilutions are 1/10.

Also, we stained the mitochondria of the untransformed mutants and the wild type strain with MitoTracker and then compared them. As shown in Fig. 4, mitochondria from the mutants look abnormal compared to the wild type, but they resemble the mitochondria of petite mutants. The fluorescent dots observed close to the plasmatic membrane in Fig. 2 may correspond to abnormal mitochondria.

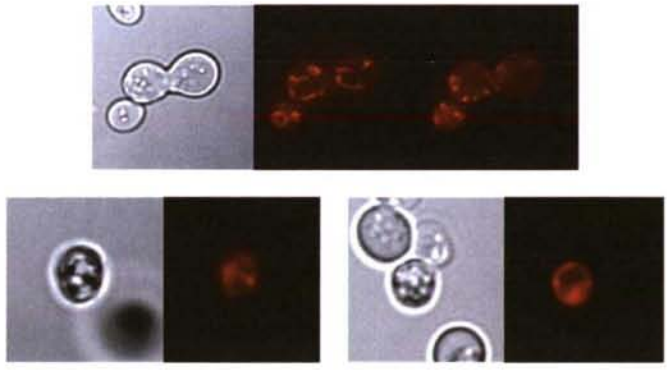

Figure 4: Fluorescence microscopy of cells stained with Mito Tracker. *S. cerevisiae* wild type mitochondria (above), *Δglr1* mutant mitochondria (below left) and *Δtrr1* mutant mitochondria (below right).

To test if Δ*glr1* and Δ*trr1* are indeed petite mutants, we cultivated both strains in fermentable and non-fermentable media (Fig. 5) and observed no differences between growth of the wild type and mutant strains. To corroborate this result, we determined the duplication times in liquid media with 2% glucose or 2% lactate as carbon sources, the wild type strain being 1.69 hours in glucose media and 3.98 hours in lactate media; the Δ*glr1* mutant 1.74 hours in glucose media and 3.39 hours in lactate media and for the Δ*trr2* mutant 1.67 hours in glucose media and 3.23 hours in lactate media. The

results revealed that these mutants do not show petite phenotype in spite of their close resemblance in morphology of their mitochondria.

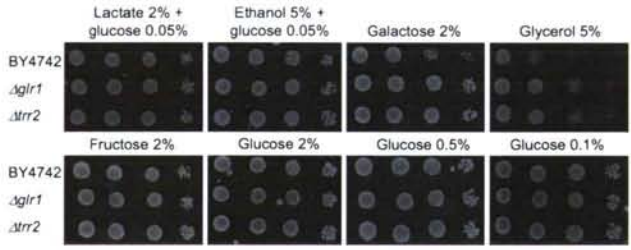

Figure 5: Growth of Δ*glr1*, Δ*trr2* and the wild type strains in several carbon sources after 2 days. The initial OD_{600} is 0.5 and the serial dilutions are 1/10.

Finally, to investigate the subcellular localization of *K. lactis* glutathione and thioredoxin reductases in a *S. cerevisiae* strain with mitochondria showing normal morphology, we transformed the BY4741 strain with the two plasmids, pKlGLR-GFP and pKlTRR1-GFP, and observed the fluorescence in a confocal microscope (Fig. 6).

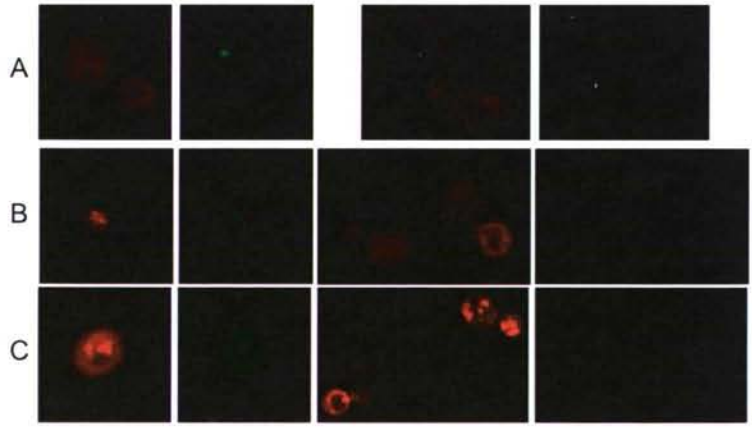

Figure 6: Expression of GFP fusions of KlGlr1p and KlTrr1p in a *S. cerevisiae* wild type strain observed under confocal microscopy. BY4741 transformed with empty pYES2-GFP (A), BY4741 transformed with pKlGLR1-GFP (B) and BY4741 transformed with pKlTRR1-GFP (C). In all cases the first and third images show the MitoTracker fluorescence and the second and fourth images show the GFP fluorescence.

The results, in spite of the low fluorescence intensity, suggest that both proteins Klglr1p and Kltrr1p seem to be localized in the cytoplasm and perhaps the glutathione reductase is also localized in the mitochondria because the highest intensity of GFP corresponds with the highest intensity of MitoTracker. The localization of Kltrr1p in the mitochondria is unclear.

Although the first AUG codon in an mRNA sequence is usually preferred as the ribosome initiation site (Kozak, 1978), the translation from downstream AUG codons may occur when the sequence context at the first AUG codon is unfavourable (Kozak, 2002). The sequences flanking each start codon, called the Kozak sequence, influence initiation efficiency (Kozak, 1984 and 1986). In yeast, the preferred consensus sequence is 5´-(A/Y)A(A/U)A-3´ with the A in position -3 being the most highly conserved of the residues surrounding the AUG codon (Cigan and Donahue, 1987). The absence of an A in position -3 of *KlGLR1* and *KlTRR1* mRNA suggests the relative poor utilization of the first AUG codon in both mRNAs and this fact may explain the poor fluorescence in the mitochondria.

The next step in our research will be to determine whether this dual targeting occurs in the cells of *K. lactis* and, if this is the case, if it is modulated by oxidative stress.

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CONCLUDING REMARKS

The main conclusions drawn from this study are summarised as follows:

1. The increment of NADPH production by the obligated utilization of the pentose phosphate pathway in *Kluyveromyces lactis* generates an increment in the transcription of genes related to the respiration and the oxidative stress response but not a clear increment in transcription of genes related to biosynthetic routes or transhydrogenase cycles.
2. The NAD- and NADPH-dependent glutamate dehydrogenase isoenzymes do not operate as a transhydrogenase cycle for cytosolic NADPH reoxidation in *K. lactis*.
3. The role of oxidative stress response in cytosolic NADPH reoxidation in *K. lactis*:
   3.1. The *KlGLR1* and *KlTRR1* genes which code for glutathione and thioredoxin reductases, respectively, have been cloned and characterized.
   3.2. Glutathione and thioredoxin reductases are not the main mechanism involved in the reoxidation of the NADPH from the pentose phosphate pathway in *K. lactis*.
   3.3. The glutathione reductase activity does not increase in response to oxidative stress and this activity decreases after the shift from aerobic to hypoxic culture conditions, having been correlated with glucose 6-phosphate dehydrogenase activity.

   Glucose 6-phosphate dehydrogenase activity does not increase in response to oxidative stress and decrease after the shift from aerobiosis to hypoxia. This activity shows a positive correlation with dissolved oxygen levels in the culture media.

   Thioredoxin reductase activity increases in response to oxidative stress and decreases after the shift from aerobiosis to hypoxia. A Klyap1p binding site is functional in the *KlTRR1* promoter.

   Catalase activity increases in response to oxidative stress and also increases after the shift from aerobiosis to hypoxia.

   The oxidative stress response is similar between the wild type and *rag2* mutant strains. The response to the shift aerobiosis/hypoxia is different; the *rag2* mutant does not grow under hypoxic conditions.

   Both glutathione reductase and catalase activities are higher in the *rag2* mutant than in the wild type strain, which is in support of the increased resistance to oxidative stress shown by the *rag2* mutant.
   3.4. Glutathione and thioredoxin reductases seem to be localized in the cytoplasm. The presence in the mitochondria of both proteins is unclear.

4. The cytosolic NADPH is mainly reoxidized by mitochondrial external alternative dehydrogenases in *K. lactis*.
   4.1. The *KlNDE1* and *KlNDE2* genes, which code for the two external alternative dehydrogenases from *K. lactis* mitochondria, have been cloned and characterized.
   4.2. *KlNDE1* and *KlNDE2* heterologous expression in *Saccharomyces cerevisiae* demonstrates that both genes code for proteins with ability to oxidize NADH and NADPH.
   4.3. A *Δklnde1* mutant was built in a *K. lactis* wild type strain, without showing a clear phenotype. This null mutant grows in fermentable and non-fermentable media with the same growth rate than the wild type, and the isolated mitochondria from both strains oxidize NADH and NADPH without great differences in the respiration rate.
   4.4. A *Δklnde1rag2* mutant was also built. This double null mutant loses the ability to grow in glucose; therefore, in the *rag2* mutant Klnde1p is essential to reoxidize the cytosolic NADPH from the pentose phosphate pathway.
   4.5. Neither pH nor calcium modulate the activity of the two mitochondrial external alternative dehydrogenases in *K. lactis*.
   The *KlNDE1* gene is not repressed by high concentration of glucose, Klnde1p activity is even up-regulated in glucose grown cells. Klnde2p activity is not down-regulated in glucose grown cells.
   Klnde1p shows higher affinity for NADPH than Klnde2p.

In sum, the external alternative dehydrogenases from *K. lactis* mitochondria, mainly Klnde1p, have the greatest capacity for the reoxidation of the cytosolic NADPH generated by the use of the pentose phosphate pathway.

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SUMMARY

The different capacities in the oxidation of the cytosolic NADPH produced by the pentose phosphate pathway represents one of the keys to the differences in the respiro-fermentative metabolism between the predominantly respiratory yeast, *Kluyveromyces lactis* and the essentially fermentative yeast, *Saccharomyces cerevisiae* (González Siso *et al.*, 2000).

When the gene that codes for the phosphoglucose isomerase (*RAG2* in *K. lactis* and *PGI1* in *S. cerevisiae*) is mutated, the glycolysis is interrupted and all the glucose has to be catabolized by the pentose phosphate pathway, generating a surplus of NADPH in the cytosol. The intermediates of the pentose phosphate pathway can be re-routed to glycolysis to be fermented or respired, thus the pentose phosphate pathway bypasses the phosphoglucose isomerase deficiency.

The *S. cerevisiae pgi1* mutant is unable to grow in media with glucose as sole carbon source due to the lack of cytosolic $NADP^+$ that blocks the pentose phosphate pathway (Boles *et al.*, 1993). The *K. lactis rag2* mutant grows in media with glucose as sole carbon source (Goffrini *et al.*, 1991), because it has more efficient mechanisms to reoxidize the cytosolic NADPH than *S. cerevisiae* (González Siso *et al.*, 1996a). These mechanisms are directly or indirectly related to the mitochondrial respiratory chain because this mutant is unable to grow in glucose in the presence of antimycin A, an inhibitor of the mitochondrial respiratory chain (Goffrini *et al.*, 1991).

There are four possible basic mechanisms of cytosolic NADPH reoxidation in yeast cells: the biosynthetic routes, the transhydrogenase cycles, the oxidative stress response and the mitochondrial external alternative dehydrogenases. In this work, we have studied the relative importance of these NADPH reoxidation mechanisms in *K. lactis*.

BIOSYNTHETIC ROUTES AND TRANSHYDROGENASE CYCLES

Two possible mechanisms for carrying out the cytosolic NADPH reoxidation could be its use in anabolic pathways and the existence of transhydrogenases. Although the existence of transhydrogenases that can transform the NADPH in NADH has not been proven in yeasts until now, the transhydrogenase activity may be derived from the operation of cycles constituted by pairs of dehydrogenase isoenzymes which catalyze the same reversible reaction but using different coenzymes, such as the glutamate dehydrogenases (Boles *et al.*, 1993) or cytoplasmic and mitochondrial alcohol dehydrogenases (Overkamp *et al.*, 2002).

In this work, we have undertaken a global study of the *K. lactis* transcriptome (wild type strain cultured in glucose, *rag2* mutant cultured in glucose and fructose)

based on the hybridization of heterologous *S. cerevisiae* DNA-arrays. This analysis, supported by other techniques, enabled us to evaluate the relative importance of these mechanisms.

We did not observe in this study any increment of transcription of any biosynthetic route involved in NADPH consumption in the *rag2* mutant cultured in glucose compared to the other cases analyzed. Nor was there any increase of expression in the *rag2* mutant cultured in glucose media of the genes involved in the glutamate dehydrogenase transhydrogenase cycle. To confirm if this cycle, whose operation restored the growth in glucose of the *S. cerevisiae pgi1* mutant (Boles *et al.*, 1993), could occur in a physiological way in *K. lactis*, Northern blotting studies of the *KlGDH1* gene, which codes for the NADPH-dependent glutamate dehydrogenase, were undertaken as well as enzymatic activity assays of the protein, comparing the *rag2* mutant with the wild type strain, both cultured in glucose. Significant differences were not observed. Therefore, we cannot conclude that this transhydrogenase cycle operates in *K. lactis*.

At the general metabolic level, the transcriptome analysis reveals, in the first place, that the pentose phosphate pathway genes transcription does not increase in the *rag2* mutant growing in glucose in comparison with the wild type strain. This fact was confirmed by Northern blotting and enzymatic activity assays and it concurs with the elevated use of pentose phosphate pathway in wild type strains from *K. lactis* (Blank *et al.*, 2005). In fact, the differences in the transcriptomes were greater between the *rag2* mutant in glucose and fructose than between the *rag2* mutant and the wild type strain, both in glucose, which is related to the main differences in the NADPH levels in the first case.

Secondly, we observed that the respiratory metabolism increased in the *rag2* mutant cultured in glucose. Specifically, the transcription of respiratory chain components, the ATPase, and related transcriptional factors rose. This corresponds to the fact that the *rag2* mutant cannot grow in glucose-antimycin A, and that this mutant exhibits a greater consumption of oxygen in glucose than the wild type strain (González Siso *et al.*, 1996a)

Thirdly, another element that stands out is the fact that the expression of some genes related to the oxidative stress response also increased in the *rag2* mutant cultured in glucose. Moreover, in the transcriptome comparison of the *rag2* mutant cultured in both carbon sources, the trehalose metabolism also appeared activated, a compound that carries out the function of protecting against oxidative stress (Alvarez Peral *et al.*, 2002). This suggested a possible greater adaptation of the *rag2* mutant to the oxidative stress, that would be related to the more respiratory metabolism because

the mitochondria is the greatest source of reactive oxygen species (ROS) in the cell (Boveris and Cadenas, 1982; Chance *et al.*, 1979). The greater adaptation to oxidative stress of the *rag2* mutant was confirmed experimentally by its greater resistance to H_2O_2 treatment. The relationship with the respiratory metabolism was confirmed by the experimental verification that *K. lactis* is more resistant than *S. cerevisiae* to oxidative stress by hydroperoxides, *K. lactis* being a yeast that predominantly uses respiration and *S. cerevisiae* a yeast that for the most part employs fermentation for catabolizing glucose .

OXIDATIVE STRESS RESPONSE ENZYMES

The third mechanism that consumes NADPH as reducing power source studied in this work is the oxidative stress response. To be exact, in this work we have focussed on the study of two enzymes, the thioredoxin reductase and the glutathione reductase, which have a central role in the defence to oxidative stress, as they are responsible for maintaining glutathione and thioredoxin reduced at the expense of the reducing power of the NADPH. Glutathione and thioredoxin are two of the most abundant cellular antioxidants necessary to maintain the redox homeostasis in the cell (Grant, 2001)

We have also studied the activity in *K. lactis* of the glucose 6-phosphate dehydrogenase, the first enzyme of the pentose phosphate pathway, since this enzyme, at least in *S. cerevisiae*, is the greatest source of NADPH needed for the reductases of the oxidative stress response (Minard and McAlister-Henn 2001).

Furthermore, the catalase activity was also studied as a control because it is a typical oxidative stress response enzyme which does not reoxidize NADPH. Therefore the catalase role in the *rag2* mutant would not be directly related to the NADPH excess that is a result of the glucose catabolization in this mutant, but rather, to the greater activity of the respiratory chain that this mutant displays (González Siso *et al.*, 1996a), the mitochondrial respiratory chain being the major source of ROS in the cell (Boveris and Cadenas, 1982; Chance *et al.*, 1979).

To ascertain the possible interrelationship between the NADPH reoxidation, the oxidative stress resistance and the respiratory metabolism, we have studied the response of the four enzymes previously cited in a *K. lactis* wild type strain and in two *rag2* mutant strains to tBOOH (ter-butyl-hydroperoxide) or H_2O_2 in flask cultures and to the shift from aerobic to hypoxic conditions (shift air/nitrogen) in fermentor cultures. Likewise, we have compared these activities in glucose cultures of both strains without any treatment.

Glucose 6-phosphate dehydrogenase

In this work, we have shown that the glucose 6-phosphate dehydrogenase is not an oxidative stress response enzyme controlled by hydroperoxides, unlike what happens with *S. cerevisiae*, whose glucose 6-phosphate dehydrogenases activity increases in response to the addition of H_2O_2 with the aim of providing NADPH (Minard and McAlister-Henn, 2001). Probably, the greater use of the pentose phosphate pathway to glucose catabolization which occurs in *K. lactis*, may be responsible for the fact that an extra increment of NADPH levels is not needed to mediate the oxidative stress response.

We have also shown that the glucose 6-phosphate dehydrogenase in *K. lactis* is an enzyme regulated by oxygen, showing a high positive correlation between its specific activity and the oxygen levels dissolved in the culture media, both in the wild type strain and in the *rag2* mutant. The difference between both strains lies in the decrease of activity produced by the shift from aerobiosis to hypoxia, a decrease that is more pronounced (approximately two times more) in the wild type strain than in the *rag2* mutant. This difference is related to the increase of the glucose flux through glycolysis, which operates in the wild type strain when the oxygen levels decrease, this increase is not possible in the *rag2* mutant due to the lack of phosphoglucose isomerase. In this modulation of the glucose flux between glycolysis and pentose phosphate pathway the keys are found that explains how it is possible that the wild type strain can grow in hypoxia but not the *rag2* mutant.

Glutathione reductase

The *KlGLR1* gene which codes for the glutathione reductase enzyme has been cloned by the DCbyPCR method which we have designed to clone genes of interest when genome partial sequences are at our disposal, from the *K. lactis* gene library in the KEp6 plasmid (Wésolowski-Louvel *et al.*, 1988). The protein coded is quite similar to the protein coded by the *S. cerevisiae GLR1* gene.

To confirm the fact that the *KlGLR1* gene codes for glutathione reductase, the clone was overexpressed in a *K. lactis* wild type strain; we observed that the activity in the transformed strain was 8 times higher than in the untransformed wild type strain.

In the N-terminal protein sequence coded by *KlGLR1*, a mitochondrial-targeting sequence appears. This single gene could have a double translation initiation site, which determines the subcellular localization of this protein in the mitochondria and cytoplasm, as occurs with the genes that code for glutathione reductase in *S. cerevisiae* (Outten and Culotta, 2004) and humans (SWISS-PROT database). Thus, preliminary subcellular localization studies were done by green fluorescent protein

(GFP) fusion and fluorescence microscopy analysis that do not let one exclude either of the two subcellular localizations for the glutathione reductase in *K. lactis*.

As far as the role in oxidative stress is concerned, *KlGLR1* transcription analysis by Northern blotting showed that the expression does not increase in response to H_2O_2 treatment. This data differs from *S. cerevisiae GLR1* expression, which increases 2.8 times its transcription on submitting the cells to an H_2O_2 concentration that is 4 times lower (Monje-Casas *et al.*, 2004). With regards to this fact, in the *KlGLR1* promoter putative binding sites for the transcriptional factor Yap1p do not exist, whereas the *S. cerevisiae GLR1* gene is regulated by this transcriptional factor (Grant *et al.*, 1996a) which mediates the induction of oxidative stress response genes. In the same way, glutathione reductase activity does not increase in *K. lactis* in response to peroxide treatment or in response to menadione which generates superoxide anions.

In contrast, the *KlGLR1* gene expressed in a multicopy plasmid can restore the *S. cerevisiae Δglr1* hydroperoxides sensibility phenotype (Trotter and Grant, 2005) so that the transformed cells grow even better than the corresponding wild type strain with tBOOH treatment. In *K. lactis* the overexpression of this gene in a wild type strain increases the resistance to tBOOH by a small amount. Regarding the greater resistance to oxidative stress of the *rag2* mutant, the glutathione reductase activity is higher in the *rag2* mutant than in the wild type strain, which suggests that glutathione reductase is involved in the greater resistance to oxidative stress showed by this mutant.

With regards to the study of its regulation by oxygen levels, the glutathione reductase activity decreases in the *K. lactis* wild strain and *rag2* mutant after the shift from aerobiosis to hypoxia.

Concerning the role of Klglr1p in the cytosolic NADPH reoxidation, in *S. cerevisiae* it has been reported that the glutathione reductase controls the glucose 6-phosphate dehydrogenase activity because it maintains the NADPH/$NADP^+$ ratio by glutathione redox balance (López-Barea *et al.*, 1990). Thus, we have analyzed the activity variation of both enzymes in *K. lactis* cells, getting a positive correlation in both wild type strain and *rag2* mutant. This suggests that in *K. lactis* the glutathione reductase takes part in the reoxidation of the NADPH produced by the pentose phosphate pathway. However, in both strains differences have been found in the glutathione reductase activity, the *rag2* mutant being higher than the wild type strain.

On the other hand, complementation studies were made in the *S. cerevisiae pgi1* mutant with the *KlGLR1* gene in an episomic plasmid, which indicates that the overexpression of this gene can restore the growth in glucose of this mutant, even in antimycin A presence. This fact suggests that the oxidation of the cytosolic NADPH

generated by the pentose phosphate pathway may be carried out by glutathione reductase at an exclusive cytosolic level in *S. cerevisiae*. In the *K. lactis rag2* mutant the overexpression of the glutathione reductase from an episomic plasmid was also carried out. In this case the Rag+ phenotype was unable to be restored, that is, the *rag2* mutant is unable to grow in glucose-antimycin A media. Thus, the NADPH reoxidation carried out by *K. lactis* glutathione reductase may be high enough to cytosolic NADPH levels from *S. cerevisiae* but not from *K. lactis*, being necessary the presence of an active mitochondrial respiratory chain in *K. lactis*.

Thioredoxin reductase

The *KlTRR1* gene was cloned by the DCbyPCR method from the *K. lactis* gene library in KEp6 (Wésolowski-Louvel *et al.*, 1988). It was revealed that this gene codes for thioredoxin reductase by purification of the protein fused to a histidine tail and specific enzyme activity measurement.

Unlike *S. cerevisiae* that has two genes: *TRR1* that codes for cytosolic tioredoxin reductase and *TRR2* that codes for mitochondrial thioredoxin reductase, *K. lactis* has a single gene coding for thioredoxin reductase. The coded protein contains a mitochondrial-targeting sequence between two methionines, which could be involved in an alternative use of two translation initiation sites. Subcellular localization experiments by fusion to GFP and fluorescence microscopy do not let know the cytosolic, mitochondrial or both compartment localizations of the Kltrr1p.

In relation to the role of Kltrr1p in the oxidative stress response in *K. lactis*, the Northern blotting analysis of *KlTRR1* gene reveals that this gene is expressed neither in the wild type strain nor in the *rag2* mutant in normal conditions. In both strains the transcription of this gene is induced by submitting the cells to H_2O_2 treatment. The induction of this gene transcription by hydroperoxides is related to the existence of a binding site for Yap1p in the *KlTRR1* promoter that we showed to be functional. Just like at the transcriptional level, the thioredoxin reductase activity increases by submitting the cells to oxidative stress by hydroperoxides.

With respect to the greater *rag2* mutant resistance to oxidative stress, there are no differences in the expression of the *KlTRR1* gene or in the thioredoxin reductase activity between the *K. lactis* wild type strain and the *rag2* mutant. Therefore, this suggests that this enzyme is not involved in the greater resistance to oxidative stress of the *rag2* mutant.

As for its regulation by oxygen levels, the activity of this enzyme acts differently between the wild type strain and the *rag2* mutant when the cells are shifted from aerobic to hypoxic conditions; in the wild type strain the activity decreases, whereas in

the *rag2* mutant, which stops growing in hypoxic conditions, the activity of this enzyme does not decrease.

In relation to its role in the NADPH reoxidation, the absence of correlation between the thioredoxin reductase activity and glucose 6-phosphate dehydrogenase activity in *K. lactis* cultured in fermentor, and the absence of differences between the wild type strain and the *rag2* mutant in *KlTRR1* transcription and enzymatic activity levels indicate that this enzyme is not involved in the NADPH reoxidation. However, the fact that this mutant does not decrease the thioredoxin reductase activity in hypoxia could be related to the use of this enzyme to reoxidize the cytosolic NADPH which is generated by the pentose phosphate pathway. To evaluate the relative importance of this reoxidation mechanism, tests were conducted to see whether the induction of the thioredoxin reductase activity by hydroperoxide treatment, could restored the growth of *rag2* mutant in glucose in presence of antimycin A. To this end, many tests were performed with different induction conditions, but on no occasion was the *rag2* mutant able to grow in glucose-antimycin A. Thus, the activity of this enzyme is not related to cytosolic NADPH reoxidation.

Catalase

The catalase activity is increased by tBOOH treatment in the wild type strain and the *rag2* mutant but this activity is higher in the *rag2* mutant than in the wild type strain in normal conditions. This fact indicates that this enzyme, like the glutathione reductase, may be related to the greater resistance to oxidative stress of the *rag2* mutant, and the adaptation to the greater activity of the respiratory chain in this mutant.

Furthermore, in the *rag2* mutant, the catalase activity does not change when the cells are shifted from aerobic to hypoxic conditions and vice-versa. In contrast, in the wild type strain this activity increases two times more at six hours after the shift from aerobiosis to hypoxia, returning to its normal levels when the culture is aerated again for three hours. This behaviour is similar to *SOD1* expression levels behaviour in *S. cerevisiae* when the cells are submitted to the same treatment (Dirmeier *et al.*, 2002), as *SOD1* codes for superoxide dismutase, another oxidative stress response enzyme.

MITOCHONDRIAL EXTERNAL ALTERNATIVE DEHYDROGENASES

The cytosolic NADPH reoxidation in *K. lactis* by mitochondrial external alternative dehydrogenases, which are non-proton-pumping, has been the first hypothesis proposed in previous studies in the laboratory to explain the ability to grow in glucose of *rag2* mutant (González Siso *et al.*, 1996a)

The publication of partial sequences of the *K. lactis* genome (Bolotin-Fukuhara *et al.*, 2000) showed a single high similar sequence to *S. cerevisiae NDE1* gene which codes for the most important external alternative dehydrogenase. The similar gene, called *KlNDE1*, was cloned by the DCbyPCR method from the *K. lactis* KEp6 gene library (Wésolowski-Louvel *et al.*, 1988) and was characterized.

Later, all the *K. lactis* genome sequences were released (http://cbi.labri.fr/Genolevures/elt/KLLA) and we found another sequence similar to Klnde1p (approximately 40%), which was more similar to the mitochondrial external NADPH calcium-dependent alternative dehydrogenase from the fungi *Neurospora crassa* (Melo *et al.*, 2001). This *K. lactis* gene, called *KlNDE2*, was cloned by PCR by means of the GAP-repair technique. The protein coded by this gene has a calcium binding motif and the characteristics to carry out the NADPH oxidation that are based on the change of an aspartate by an arginine about ten aminoacid residues downstream of the first glycine-rich motif for coenzyme binding (Lesk, 2001)

An expression study of the *KlNDE1* gene by Northern blotting was undertaken. The transcription increased 2.4-fold in the *rag2* mutant cultured in glucose in comparison with the wild type strain. Moreover, in this study we also observed that the *KlNDE1* induction in the *rag2* mutant cultured in glucose disappears when the cells are submitted to oxidative stress which increases the transcription of other genes coding for enzymes that use NADPH, such as the *KlTRR1* gene. This was the first indication that supported the hypothesis of the ability of Klnde1p to reoxidize NADPH. Overkamp *et al.*, (2002) compared the NADH and NADPH respiration rates by isolated mitochondria from the *K. lactis* wild type strain and the *rag2* mutant, thus obtaining an induction in the mutant of 2.6-fold for NADPH and 3.2-fold for NADH. These levels of induction correspond to the increase in the *KlNDE1* gene transcription levels that we found in the expression study between the *rag2* mutant and the wild type strain. These data seemed to indicate that the increase of both *KlNDE1* transcription and protein activity were mainly responsible for the growth of the *rag2* mutant in glucose. This hypothesis was confirmed upon the construction of the double mutant, *Δklnde1rag2*, which not only lost the ability to grow in glucose, but also ended up being practically unviable. Consequently, the *KlNDE1* gene is essential for the growth of the *rag2* mutant in glucose and this fact also implies that neither the glutathione reductase nor any other enzymes with the ability to reoxidize NADPH are efficient enough to compensate for the lack of Klnde1p.

To confirm the fact that the proteins Klnde1p and Klnde2p can truly oxidize NADPH and to ascertain if these proteins oxidized NADH, the *S. cerevisiae Δnde1nde2* strain was built, being the isolated mitochondria from this double mutant unable to

oxidize NADH and NADPH. The double mutant was transformed separately with the two clones that carried *KlNDE1* and *KlNDE2*, and in both cases the isolated mitochondria from the transformants acquired the capacity for oxidizing NADH and NADPH with the same efficacy. Therefore, Klnde1p and Klnde2p oxidize both coenzymes.

To study the role of these genes in their own cellular environments, the null mutant in the first external alternative dehydrogenase, *Δklnde1*, was created in a *K. lactis* wild type strain. This mutant did not show any differences in the rate of growth compared to the wild type strain neither in non-fermentable nor in fermentable carbon sources. Surprisingly, we did not observe decreases in the oxidation rates of NADH and NADPH by isolated mitochondria of this mutant compared to the wild type strain. To ascertain what caused the *Δklnde1rag2* mutant inability to grow in glucose if this double mutant contained an intact *KlNDE2* gene, we have studied several possible factors.

Given that the optimal activity of the two external alternative dehydrogenases from *N. crassa* is found in a different pH range (Melo *et al.*, 2001), we assayed if this fact occurred in the enzymes of *K. lactis*. To this end, we used isolated mitochondria from the wild type strain and the *Δklnde1* mutant, and the result was that the optimal activity of the two external alternative dehydrogenases is found in the same pH range, for both NADH as well as for NADPH as substrates.

We also studied the glucose catabolic repression of the *KlNDE1* gene by Northern blotting in a wild type strain cultured in different concentrations of glucose and lactate, which showed that this type of regulation in the *KlNDE1* gene does not exist. Nor did we observe a reduction in the rate of oxidation of NADH or NADPH, either in isolated mitochondria from the wild type strain or the *Δklnde1* mutant when both of them are cultured in glucose compared to lactate. Therefore we can conclude that down-regulation by glucose does not operate in the two external alternative dehydrogenases of *K. lactis*. However, the NADH and NADPH oxidation rates by isolated mitochondria from the wild type strain increase when the cells are cultivated in glucose compared to lactate. This fact also occurs in the NADH oxidation rate by the *Δklnde1* isolated mitochondria but not in the NADPH oxidation rate. Consequently, the results suggest that an up-regulation by glucose operates mainly for Klnde1p, this being the necessary enzyme so as to permit the *rag2* mutant to grow in glucose.

Thirdly, some cytosolic parameters may be modulating the activity of these two enzymes in *K. lactis*, making necessary the Klnde1p presence for the growth of the *rag2* mutant. Møller (2001) describes how the activity of the alternative

dehydrogenases of plants is regulated by two cytosolic parameters, substrate concentration and calcium. In this thesis we have studied the role of both parameters in the regulation of the two external alternative dehydrogenases from *K. lactis*.

We have based the experiments in the characterization data of *N. crassa* Nde1p (Melo *et al.*, 2001) to assay the possible regulation by calcium of the Klnde2p activity in *S. cerevisiae Δnde1nde2* isolated mitochondria transformed with *KlNDE2* gene and in *K. lactis Δklnde1* mutant isolated mitochondria, and we did not observe any variations in its activity. Thus, we conclude that calcium does not regulate the activity of this enzyme.

To study the influence of the substrate concentration, we have conducted oxygen consumption assays by isolated mitochondria from the wild type strain and the *Δklnde1* mutant at different NADPH concentration levels, with the data obtained that conform to Michaelis-Menten kinetics, we have been able to determine K_M and V_{max} kinetic parameters. The K_M is 2-fold lower in the wild type strain isolated mitochondria than in the *Δklnde1* isolated mitochondria. Therefore, Klnde1p shows a higher affinity for NADPH than Klnde2p, which implies that the cytosolic NADPH concentration may modulate the activity of both enzymes. The difference in the K_M of Klnde1p and Klnde2p is one of the mechanisms that explain why the *Δklnde1rag2* mutant cannot grow in glucose. This assertion is supported by the overexpression of the genes which code for these proteins in the *S. cerevisiae pgi1* mutant, whereas the *KlNDE1* gene restores the growth of this mutant in media with 2% glucose, the *KlNDE2* gene only allows the growth of this mutant in media with up to 0.4% glucose, which indicates that when the glucose flux through the pentose phosphate pathway is high, the NADPH oxidation by Klnde2p is not high enough so that the lack of $NADP^+$ blocks the pentose phosphate pathway, preventing the *pgi1* mutant to grow in glucose. Moreover, the necessity of a quick NADPH reoxidation would still be more pronounced in *K. lactis* than in *S. cerevisiae* due to the fact that the pentose phosphate pathway activity is higher in *K.lactis* than in *S. cerevisiae*.

In conclusion, two factors explain the inability of the *Δklnde1rag2* mutant to grow in glucose: the different affinity of the external alternative dehydrogenases of *K. lactis* for the NADPH, Klnde1p being the greater of the two, and the positive regulation by glucose which acts on this protein.

References (pp. 173-183)

RESUMEN

La diferente capacidad en la oxidación del NADPH citosólico producido por la ruta de las pentosas fosfato representa una de las claves en las diferencias en el metabolismo respiro-fermentativo entre la levadura preferentemente respiradora *Kluyveromyces lactis* y la levadura preferentemente fermentadora *Saccharomyces cerevisiae* (González Siso *et al.*, 2000).

Al mutar el gen que codifica para la fosfoglucosa isomerasa (*RAG2* en *K. lactis*, *PGI1* en *S. cerevisiae*), la glicólisis se interrumpe y toda la glucosa tiene que ser catabolizada por la ruta de las pentosas fosfato produciendo un exceso de NADPH en el citosol. Los intermediarios de la ruta de las pentosas fosfato pueden redirigirse a la glicólisis para ser fermentados o respirados, de modo que la ruta de las pentosas fosfato hace de *bypass* de la deficiencia en fosfoglucosa isomerasa.

El mutante *pgi1* de *S. cerevisiae* es incapaz de crecer en medios con glucosa como única fuente de carbono debido a una carencia de NADP que bloquea la ruta de las pentosas fosfato (Boles *et al.* 1993). El mutante *rag2* de *K. lactis* crece en medios con glucosa como única fuente de carbono (Goffrini *et al.*, 1991) gracias a que posee mecanismos más eficientes para reoxidar el NADPH citosólico (González Siso *et al.*, 1996a). Estos mecanismos están directa o indirectamente relacionados con la cadena respiratoria, ya que este mutante es incapaz de crecer en glucosa en presencia del inhibidor de la cadena respiratoria antimicina A (Goffrini *et al.*, 1991)

En las células de levaduras podrían existir cuatro posibles mecanismos básicos de reoxidación del NADPH citosólico: las rutas biosintéticas, los ciclos transhidrogenasa, la respuesta a estrés oxidativo y las deshidrogenasas alternativas externas mitocondriales. El objetivo principal de este trabajo es estudiar la importancia relativa de estos mecanismos de reoxidación del NADPH en *K. lactis*.

Para desarrollar este trabajo, las líneas celulares de bacterias y levaduras fueron cultivadas y mantenidas en los medios apropiados, según indicaciones de Sambrook *et al.* (1989) y Kaiser *et al.* (1994). Para las técnicas básicas de biología molecular, se aplicaron los protocolos descritos por Sambrook *et al.* (1989), Rose *et al.* (1990) y Kaiser *et al.* (1994), con las adaptaciones pertinentes según la práctica habitual del laboratorio. Respecto a los experimentos de actividades enzimáticas se utilizaron los protocolos descritos por: Smith *et al.* (1988), Kuby y Noltmann (1966), Holmgren y Björnstedt (1995) y Aebi (1984). Finalmente, para el aislamiento de mitocondrias, se aplicó el protocolo de Herrmann *et al.* (1994) y para el estudio de consumo de oxígeno en preparaciones mitocondriales, el método descrito por Luttik *et al.* (1998).

RUTAS BIOSINTÉTICAS Y CICLOS TRANSHIDROGENASA

Dos posibles mecanismos para llevar a cabo la reoxidación del NADPH citosólico serían su uso en determinadas rutas anabólicas y la existencia de transhidrogenasas. Aunque la existencia de transhidrogenasas que sean capaces de transformar el NADPH en NADH no ha sido demostrada hasta el momento en levaduras, la actividad transhidrogenasa puede derivar del funcionamiento de ciclos constituidos por pares de isoenzimas que catalizan la misma reacción reversible pero usando coenzimas distintos, como las glutamato deshidrogenasas (Boles *et al.*, 1993) o las alcohol deshidrogenasas citoplasmáticas y mitocondriales (Overkamp *et al.*, 2002).

En este trabajo realizamos un estudio global del transcriptoma de *K. lactis* (cepa silvestre cultivada en glucosa, mutante *rag2* cultivado en glucosa y fructosa) basado en la hibridación de *arrays* heterólogos de *S. cerevisiae* que, apoyado por otras técnicas, permitió valorar la importancia relativa de estos mecanismos.

En este estudio no se observó aumento en la expresión de genes de ninguna ruta biosintética implicada en el consumo de NADPH en el mutante *rag2* cultivado en glucosa frente a los otros dos casos analizados. Tampoco apareció aumento de expresión de los genes implicados en el ciclo transhidrogenasa de la glutamato deshidrogenasa en el mutante *rag2* cultivado en glucosa. El funcionamiento de este ciclo restaura el crecimiento en glucosa del mutante *pgi1* de *S. cerevisiae* (Boles *et al.* 1993). Para comprobar si este ciclo funciona de manera fisiológica en *K. lactis*, se realizaron estudios de *Northern blotting* del gen *KlGDH1* (que codifica para la glutamato deshidrogenasa dependiente de NADPH) y ensayos de actividad enzimática de su proteína, tanto en el mutante *rag2* como en la cepa silvestre ambos cultivados en glucosa y en fructosa. No se observaron diferencias importantes, por lo que no podemos concluir que este ciclo opere en *K. lactis*.

A nivel metabólico general, del análisis de los transcriptomas destaca, en primer lugar, que en comparación con la cepa silvestre, en el mutante *rag2* creciendo en glucosa no se observó un incremento en la transcripción de genes de la ruta de las pentosas fosfato. Este dato fue comprobado para la enzima glucosa 6-fosfato deshidrogenasa por *Northern blotting* y por ensayos de actividad enzimática. Estos resultados concuerdan con el elevado uso de la ruta de las pentosas fosfato en cepas silvestres de *K. lactis* (Blank *et al.*, 2005). De hecho, las diferencias en los transcriptomas fueron mayores entre el *rag2* cultivado en glucosa y fructosa que entre el *rag2* y la cepa silvestre cultivados en glucosa, lo que se relaciona con mayores diferencias en los niveles de NADPH citosólico en el primer caso.

En segundo lugar, se observó un aumento del metabolismo respirador en el mutante *rag2* en glucosa. En concreto, aumentó la transcripción de componentes de la cadena respiratoria, de la ATPasa y factores transcripcionales relacionados. Esto concuerda con el hecho de que el mutante *rag2* no pueda crecer en glucosa-antimicina A (Goffrini *et al.*, 1991) y de que presente un mayor consumo de oxígeno en cultivos en glucosa que la cepa silvestre (González Siso *et al.*, 1996a).

En tercer lugar, también destaca el aumento de expresión de algunos genes de respuesta a estrés oxidativo en el mutante *rag2* en glucosa. En la comparación del transcriptoma del mutante *rag2* en las dos fuentes de carbono apareció activado también el metabolismo de la trehalosa, un compuesto que desempeña una función de protección frente al estrés oxidativo (Alvarez-Peral *et al.*, 2002). Esto sugirió una posible mayor adaptación del mutante *rag2* al estrés oxidativo, lo cual podría estar relacionado con su metabolismo más respirador puesto que la mitocondria es la mayor fuente de especies de oxígeno reactivas (ROS) en la célula (Boveris y Cadenas, 1982; Chance *et al.*, 1979). La mayor adaptación al estrés oxidativo del mutante *rag2* frente a la cepa silvestre se confirmó experimentalmente por su mayor resistencia al tratamiento con H_2O_2. La relación con el metabolismo más respirador se corroboró por la constatación experimental de que *K. lactis* es más resistente que *S. cerevisiae* al estrés oxidativo mediado por peróxidos, siendo *K. lactis* una levadura que usa preferentemente la respiración y *S. cerevisiae* una levadura que usa preferentemente la fermentación para catabolizar la glucosa.

ENZIMAS DE RESPUESTA A ESTRÉS OXIDATIVO

El tercer mecanismo estudiado en este trabajo que consume NADPH como fuente de poder reductor es la respuesta a estrés oxidativo. En concreto, nos hemos centrado en el estudio de dos enzimas, la tiorredoxina reductasa y la glutatión reductasa, las cuales desempeñan un papel central en la respuesta frente al estrés oxidativo, siendo las encargadas de mantener, a expensas del poder reductor del NADPH, los niveles de glutatión y tiorredoxinas reducidas, dos de los más abundantes antioxidantes celulares encargados de mantener la homeostasis redox en la célula (Grant, 2001).

También hemos estudiado la actividad en *K. lactis* de la glucosa 6-fosfato deshidrogenasa, la primera enzima de la ruta de las pentosas fosfato, ya que ésta, al menos en *S. cerevisiae*, es la mayor fuente del NADPH necesario para las reductasas de defensa a estrés oxidativo (Minard y McAlister-Henn, 2001).

Además, hemos utilizado el comportamiento de la enzima catalasa como control, dado que es una enzima típica de respuesta a estrés oxidativo que no reoxida NADPH y por tanto su papel en el mutante *rag2* no estaría directamente relacionado con el exceso de NADPH, sino más bien con la mayor actividad de la cadena respiratoria que presenta (González Siso *et al.*, 1996a), siendo la cadena respiratoria la mayor fuente de ROS en la célula (Boveris y Cadenas, 1982; Chance *et al.*, 1979)

Para averiguar la posible interrelación entre la reoxidación del NADPH, la resistencia al estrés oxidativo y el metabolismo respirador, hemos realizado medidas de actividad de las cuatro enzimas citadas, en una cepa silvestre de *K. lactis* y en dos cepas mutantes *rag2*, para estudiar la respuesta tanto al tratamiento con tBOOH (ter-butil-hidroperóxido) o H_2O_2 en cultivos en matraz, como al paso de condiciones aeróbicas a hipóxicas (cambio de aire por nitrógeno) en cultivos en fermentador. Asimismo, hemos comparado dichas actividades en cultivos en glucosa de ambas cepas sin ningún tratamiento.

Glucosa 6-fosfato deshidrogenasa

En este trabajo hemos demostrado que la glucosa 6-fosfato deshidrogenasa en *K. lactis* no es una enzima de respuesta a estrés mediado por hidroperóxidos, a diferencia de lo que ocurre en *S. cerevisiae*. Probablemente la mayor utilización de la ruta de las pentosas fosfato para la catabolización de la glucosa que ocurre en levaduras respiradoras, entre las cuales se encuentra *K. lactis* (Blank *et al.*, 2005), sea la responsable de que no se precise un aumento extra de los niveles de NADPH para mediar la respuesta a estrés oxidativo.

También hemos demostrado que la glucosa 6-fosfato deshidrogenasa en *K. lactis* es una enzima regulada positivamente por oxígeno, tanto en la cepa silvestre como en el mutante *rag2*. La diferencia entre ambas cepas reside en el descenso de actividad que se produce al pasar de condiciones de cultivo aeróbicas a hipóxicas, descenso que es más acusado (aproximadamente el doble) en la cepa silvestre. Esta diferencia se relaciona con el desvío del flujo de glucosa desde la ruta de las pentosas fosfato hacia la glucólisis. Este desvío del flujo de glucosa opera en la cepa silvestre al disminuir los niveles de oxígeno disuelto en el medio de cultivo y no es posible en el mutante rag2 debido a la carencia en fosfoglucosa isomerasa. En consecuencia, el mutante rag2 no puede crecer en hipoxia.

Glutatión reductasa

El gen que codifica para la enzima glutatión reductasa (*KlGLR1*) se ha clonado por el método DCbyPCR, que hemos diseñado para clonar genes de interés cuando se dispone de secuencias parciales del genoma, a partir de una genoteca de *K. lactis*

en el plásmido KEp6 (Wésolowski-Louvel *et al.*, 1988). La proteína que codifica posee alta similitud con la proteína que codifica el gen *GLR1* en *S. cerevisiae*.

Para corroborar que este gen codifica para la enzima glutatión reductasa se sobreexpresó el clon en una cepa silvestre de *K. lactis*, observándose que la actividad enzimática específica de la cepa transformada aumentaba 8 veces sobre los valores de la cepa sin transformar.

En el extremo amino terminal de la secuencia proteica que codifica el gen *KlGLR1* aparece una secuencia de transporte a la mitocondria. Este único gen podría tener un doble sitio de inicio de la traducción que determinase la localización subcelular de la proteína en el citoplasma o en la mitocondria, al igual que ocurre en los genes que codifican para la glutatión reductasa de *S. cerevisiae* (Outten y Culotta, 2004) y de humanos (base de datos SWISS-PROT). Por tanto, se han hecho estudios preliminares de localización subcelular mediante fusión a la proteína fluorescente verde (GFP) y análisis en microscopía de fluorescencia que no permiten excluir ninguna de las dos sublocalizaciones para la glutatión reductasa de *K. lactis*.

En relación a su papel en la respuesta a estrés oxidativo, el análisis de la transcripción mediante *Northen blotting* del gen *KlGLR1* mostró que su expresión no aumenta frente al tratamiento con H_2O_2. Este dato difiere del comportamiento del gen *GLR1* de *S. cerevisiae*, el cual aumenta 2,8 veces su transcripción al tratar las células con una concentración 4 veces menor de H_2O_2 (Monje-Casas *et al.*, 2004). Relacionado con este hecho, en el promotor del gen *KlGLR1* no existe el hipotético sitio de unión al factor transcripcional Yap1p, mientras que en el promotor del gen de *S. cerevisiae GLR1* hay una diana de este factor transcripcional (Grant *et al.*, 1996a) que media la inducción de genes de respuesta a estrés oxidativo. Del mismo modo, la actividad específica glutatión reductasa no aumenta en *K. lactis* ni frente al tratamiento con peróxidos ni mediante otro tipo de oxidante como la menadiona, que genera aniones superóxido.

Por otro lado, el gen *KlGLR1* expresado en un plásmido multicopia puede revertir el fenotipo de sensibilidad a hidroperóxidos del mutante *Δglr1* de *S. cerevisiae* (Trotter y Grant, 2005), haciendo crecer las células incluso mejor que su cepa silvestre con tratamiento con tBOOH. En *K. lactis* la sobreexpresión de este gen en una cepa silvestre también aumenta un poco su resistencia a tBOOH. En lo que concierne a la mayor resistencia frente al estrés oxidativo del mutante *rag2*, existe un aumento de los niveles de actividad glutatión reductasa en la cepa mutante *rag2* con respecto a la cepa silvestre de *K. lactis*, lo que sugiere que la glutatión reductasa está implicada en la mayor resistencia al estrés oxidativo mostrada por el mutante *rag2*.

En relación al estudio de su regulación frente a los niveles de oxígeno, la actividad glutatión reductasa disminuye tanto en la cepa silvestre de *K. lactis* como en el mutante *rag2* al pasar de condiciones de cultivo aeróbicas a hipóxicas. También la actividad glutatión reductasa de *Kluyveromyces marxianus* presenta una mayor respuesta frente a altas concentraciones de oxígeno que frente a otro tipo de estrés como el generado por hidroperóxidos o paraquat (Pinheiro *et al.*, 2002).

Respecto al papel de Klglr1p en la reoxidación del NADPH, en *S. cerevisiae* se ha descrito que la glutatión reductasa controla la actividad de la glucosa 6-fosfato deshidrogenasa porque mantiene la adecuada relación NADPH/NADP a través del balance redox del glutatión (López-Barea *et al.*, 1990). Por tanto, analizamos conjuntamente la variación de la actividad de ambas enzimas en células de *K. lactis* en cultivo en fermentador obteniendo una correlación positiva tanto para la cepa silvestre como para el mutante *rag2*, lo que sugiere que en *K. lactis* la glutatión reductasa participa en la reoxidación del NADPH producido por la ruta de las pentosas. Sin embargo, entre ambas cepas se vuelven a manifestar diferencias en la actividad glutatión reductasa, ya que a la misma actividad glucosa 6-fosfato deshidrogenasa, la actividad glutatión reductasa es mayor en el mutante *rag2* que en la cepa silvestre.

También se han hecho estudios de complementación en el mutante de *S. cerevisiae pgi1* con el gen *KlGLR1* en un plásmido episómico, que indican que la sobreexpresión de dicho gen puede revertir el fenotipo de esta cepa haciéndola crecer en glucosa. La reversión de este fenotipo también se consigue en presencia de antimicina A. Este hecho sugiere que la reoxidación del exceso de NADPH generado por la ruta de las pentosas fosfato puede ser llevada a cabo por la enzima glutatión reductasa a nivel exclusivamente citosólico en *S. cerevisiae*.

En el mutante de *K. lactis rag2* también se ha llevado a cabo la sobreexpresión de la glutatión reductasa mediante un plásmido episómico, en este caso no se ha conseguido restaurar el fenotipo Rag^+, es decir, el crecimiento en glucosa con antimicina A. Esto puede ser debido al mayor uso de la ruta de las pentosas fosfato por parte de *K. lactis* en comparación con *S. cerevisiae*, que conlleva una mayor generación de NADPH, de modo que la reoxidación del NADPH por la glutatión reductasa puede ser suficiente para los niveles presentes en *S. cerevisiae* pero no en *K. lactis*, siendo necesaria en esta levadura la intervención de la cadena respiratoria.

Tiorredoxina reductasa

Se ha clonado el gen *KlTRR1* por el método DCbyPCR a partir de la genoteca de *K. lactis* en KEp6 (Wésolowski-Louvel *et al.*, 1988). Se probó que este gen codificaba para la enzima tiorredoxina reductasa mediante la purificación de la proteína fusionada a una cola de histidinas y la medida de su actividad específica,

debido a que la fuerte regulación a la que está sometido este gen no permite su sobreexpresión en la célula.

A diferencia de *S. cerevisiae* que posee dos genes: *TRR1* que codifica para la tiorredoxina reductasa citoplasmática y *TRR2* que codifica para la enzima mitocondrial, *K. lactis* sólo posee un gen que codifica para la tiorredoxina reductasa. En el extremo amino terminal de esta proteína aparece una secuencia de transporte a la mitocondria y dos metioninas que podrían estar relacionadas con un uso alternativo de codón de inicio de la traducción. Experimentos de localización subcelular mediante fusión a GFP y microscopía de fluorescencia no permitieron dilucidar la localización citosólica, mitocondrial o en ambos compartimentos de la proteína Kltrr1p.

En relación a su papel en la respuesta a estrés oxidativo en *K. lactis*, el análisis de *Northern blotting* del gen *KlTRR1* mostró que este gen no se expresa ni en la cepa silvestre ni en el mutante *rag2* en ausencia de estrés oxidativo. En ambas cepas se induce la transcripción de este gen al someterlas a un tratamiento con H_2O_2. La inducción de la transcripción de este gen por hidroperóxidos está relacionada con la existencia en el promotor de un sitio de unión al factor transcripcional Yap1p que hemos demostrado que es funcional. Al igual que a nivel transcripcional, la actividad tiorredoxina reductasa aumenta al someter a las células a estrés oxidativo mediado por peróxidos.

En lo que concierne a la mayor resistencia del mutante *rag2* frente al estrés oxidativo, no existen diferencias de expresión en el gen *KlTRR1* ni en la actividad enzimática tiorredoxina reductasa entre la cepa silvestre y el mutante *rag2* de *K. lactis*. Por tanto, esto sugiere que esta enzima no está implicada en la mayor resistencia al estrés oxidativo del mutante *rag2*.

En relación al estudio de su regulación frente a los niveles de oxígeno, esta enzima se comporta de forma distinta en la cepa silvestre que en el mutante *rag2* de *K. lactis* al pasar de condiciones aeróbicas a hipóxicas, en la cepa silvestre la actividad disminuye, mientras que en el mutante *rag2*, en el cual cesa su crecimiento en condiciones hipóxicas, la actividad de esta enzima no disminuye.

Con relación a su papel en la reoxidación del NADPH, la ausencia de correlación entre la actividad tiorredoxina reductasa y la glucosa 6-fosfato deshidrogenasa en células de *K. lactis*, y la ausencia de diferencias entre la cepa salvaje y el mutante *rag2*, tanto en el análisis de trascripción del gen como en los ensayos de actividad enzimática tiorredoxina reductasa, indican que esta enzima no está implicada en la reoxidación del NADPH. Sin embargo, el hecho de que en el mutante *rag2* no disminuya la actividad tiorredoxina reductasa en hipoxia podría relacionarse con su utilización para reoxidar el NADPH citosólico que se genera en la

ruta de las pentosas fosfato. Para valorar la importancia relativa de este mecanismo de reoxidación, se determinó si aumentando la actividad de esta enzima mediante estrés oxidativo generado por tBOOH en el mutante *rag2*, se podría restaurar el crecimiento en glucosa en presencia de antimicina A. Para ello se hicieron numerosas pruebas a distintas concentraciones del oxidante y con diversos pases de las células por condiciones de estrés, pero en ningún caso el mutante *rag2* era capaz de crecer en glucosa en presencia de antimicina A. Por tanto, la actividad de esta enzima no está relacionada con la reoxidación del NADPH citosólico.

Catalasa

El tratamiento con tBOOH aumenta la actividad catalasa tanto en la cepa silvestre como en el mutante *rag2* de *K. lactis*, pero existen diferencias entre ambas cepas en condiciones de ausencia de estrés oxidativo, siendo esta actividad más elevada en el mutante. Este hecho indica que esta enzima, al igual que la glutatión reductasa, puede estar relacionada con la mayor resistencia del mutante *rag2* al estrés oxidativo, y que su actividad incrementada puede ser debida a una adaptación a la mayor actividad de la cadena respiratoria del mutante *rag2*.

Además, en el mutante *rag2* la actividad catalasa no sufre variación al cambiar las condiciones de cultivo de aeróbicas a hipóxicas y viceversa. En contraposición, en la cepa silvestre esta actividad aumenta más de dos veces a las 6 horas de tratamiento con nitrógeno de un cultivo iniciado en condiciones aeróbicas, volviendo a sus niveles normales de actividad al airear de nuevo los cultivos hipóxicos durante 3 horas. Este comportamiento es similar al que opera sobre los niveles de mRNA del gen *SOD1* de *S. cerevisiae,* que codifica para la superóxido dismutasa, cuando se someten las células al mismo tratamiento (Dirmeier *et al.*, 2002)

DESHIDROGENASAS MITOCONDRIALES ALTERNATIVAS EXTERNAS

La reoxidación del NADPH citosólico en *K. lactis* por parte de deshidrogenasas mitocondriales alternativas externas, que se caracterizan por no bombear protones, ha sido la primera hipótesis formulada en trabajos previos del laboratorio para explicar la capacidad de crecimiento en glucosa del mutante *rag2* (González Siso *et al.*, 1996a).

La publicación de secuencias parciales del genoma de *K. lactis* (Bolotin-Fukuhara *et al.*, 2000), mostró una única secuencia altamente similar a la del gen *NDE1* de *S. cerevisiae*, que codifica para la deshidrogenasa alternativa externa más importante. Mediante el método DCbyPCR se clonó el gen de *K. lactis* a partir de una genoteca en KEp6 (Wésolowski-Louvel *et al.*, 1988) y se caracterizó. Este gen se denominó *KlNDE1*.

Posteriormente, tras la liberación de la totalidad de secuencias del genoma de *K. lactis* (http://cbi.labri.fr/Genolevures/elt/KLLA), al someter la proteína Klnde1p a un *BLAST* encontramos una secuencia con una similitud de aproximadamente un 40%, que era más similar a la NADPH deshidrogenasa mitocondrial alternativa externa dependiente de calcio del hongo *Neurospora crassa* (Melo *et al.*, 2001). Este gen de *K. lactis* se clonó por PCR mediante la técnica de recombinación *gap-repair*. La proteína codificada por este gen poseía un motivo de unión a calcio y las características para realizar la reoxidación del NADPH, las cuales se basan en el cambio de un aspartato por una arginina a unos 10 residuos aminoacídicos hacia la zona carboxilo-terminal del primer motivo rico en glicinas para la unión al coenzima (Lesk, 2001). A este nuevo gen se le denominó *KlNDE2*.

Se realizó un estudio de expresión mediante *Northern blotting* del gen *KlNDE1*, dónde se observó que la transcripción de este gen aumentaba 2,4 veces en el mutante *rag2* cuando era cultivado en glucosa en comparación con la cepa silvestre cultivada en las mismas condiciones. Además, en este estudio también se observa que la inducción de la transcripción en el mutante *rag2* en glucosa desaparece al someter a las células a estrés oxidativo, lo que aumenta la transcripción de otros genes que codifican enzimas que consumen NADPH, cómo el gen *KlTRR1*. Este fue el primer indicio que apoyaba la hipótesis de la capacidad de Klnde1p de reoxidar NADPH. Overkamp y colaboradores, en el año 2002, compararon las medidas de respiración de NADPH y NADH por mitocondrias aisladas de la cepa silvestre y del mutante *rag2* de *K. lactis* obteniendo una inducción en el mutante de 2,6 veces en el ratio de respiración para el NADPH y de 3,2 veces para el NADH, estos niveles de inducción de respiración se corresponden con el aumento de la transcripción del gen *KlNDE1* que nosotros encontramos en el mutante *rag2* con respecto a la cepa silvestre. Estos datos parecían indicar que el aumento de transcripción del gen *KlNDE1* y de la actividad de la proteína era el principal responsable del crecimiento en glucosa del mutante *rag2*. Esta hipótesis se confirmó con la construcción del doble nulo *Δklnde1rag2* que no sólo perdió la capacidad de crecer en glucosa sino que resultó ser prácticamente inviable. En consecuencia, el gen *KlNDE1* es imprescindible para el crecimiento del mutante *rag2* en glucosa, lo que apoya la hipótesis de que las deshidrogenasas mitocondriales alternativas externas son fundamentales para la reoxidación del NADPH citosólico. Además, esto implica que ni la glutatión reductasa ni ninguna otra enzima con capacidad para reoxidar el NADPH es lo suficientemente eficaz para compensar la falta de Klnde1p.

Para corroborar que efectivamente las proteínas Klnde1p y Klnde2p podían reoxidar NADPH y para averiguar si reoxidaban NADH, se construyó el doble nulo de

S. cerevisiae Δnde1nde2, el cual no poseía capacidad de oxidación detectable ni de NADH ni de NADPH. Este doble nulo fue transformado por separado con los dos clones que llevaban los genes que codificaban para las deshidrogenasas alternativas externas de *K. lactis*. En ambos casos los transformantes adquieren la capacidad de oxidar tanto NADH como NADPH con la misma eficacia. Por tanto, Klnde1p y Klnde2p oxidan los dos coenzimas.

Para poder estudiar el comportamiento de estos genes en su propio entorno celular se construyó el mutante nulo en la primera deshidrogenasa mitocondrial externa caracterizada en una cepa silvestre de *K. lactis* (*Δklnde1*). Dicho mutante no mostró variación de la velocidad de crecimiento con respecto a la cepa silvestre en medios con distintas fuentes de carbono, fermentables y no fermentables. Sorprendentemente, no observamos disminución en la oxidación del NADPH ni del NADH por mitocondrias aisladas de este mutante frente a la cepa silvestre. Para investigar cual era la causa de que el doble mutante *Δklnde1rag2* no creciese en glucosa si disponía del gen *KlNDE2* intacto, hemos estudiado varios posibles factores.

Dado que la actividad óptima de las dos deshidrogenasas externas de *Neurospora crassa* se encuentra en un rango de pH diferente (Melo *et al.*, 2001), ensayamos si esto ocurría con las dos enzimas de *K. lactis*, para lo que utilizamos mitocondrias aisladas de la cepa silvestre y del mutante *Δnde1*, con el resultado de que la actividad óptima de las dos enzimas se encuentra en el mismo rango de pH, tanto con NADH como con NADPH como sustratos

Con respecto a la represión catabólica por glucosa, se han hecho ensayos de *Northern blotting* en una cepa silvestre cultivada en distintas concentraciones de glucosa (fuente de carbono fermentable) y en lactato (fuente de carbono no fermentable), que demostraron que no existía este tipo de regulación en el gen *KlNDE1*. Tampoco se observó menor velocidad de oxidación del NADPH ni del NADH tanto en las mitocondrias aisladas de la cepa silvestre como del mutante *Δnde1* cuando ambos son cultivados en glucosa en comparación a cuando son cultivados en lactato. Por tanto, podemos concluir que no existe regulación negativa por glucosa en ninguna de las dos deshidrogenasas alternativas externas de *K. lactis*. Sin embargo, el ratio de oxidación de NADH y NADPH por mitocondrias aisladas aumenta cuando la cepa silvestre es cultivada en glucosa frente a cuando es cultivada en lactato, este hecho también ocurre en las mitocondrias aisladas procedentes del mutante *Δklnde1* para el ratio de oxidación del NADH pero no para el ratio de oxidación del NADPH. En consecuencia, los resultados sugieren que existe una regulación positiva por glucosa que opera fundamentalmente sobre Klnde1p, siendo esta la enzima necesaria para

que el mutante *rag2* pueda crecer en glucosa, aunque el mecanismo regulador no haya sido identificado.

Basándonos en los datos de caracterización de Nde1p de *N. crassa* (Melo *et al.*, 2001), hemos ensayado la posible regulación por calcio de la actividad Klnde2p en mitocondrias aisladas del doble nulo *Δnde1nde2* de *S. cerevisiae* transformado con el gen *KlNDE2* y en mitocondrias aisladas del mutante *Δnde1* de *K. lactis*, sin observar ningún tipo de variación en su actividad. Por tanto, podemos concluir que el calcio no regula la actividad de esta enzima.

Para estudiar la influencia de la concentración de substrato, hemos realizado ensayos de consumo de oxigeno por mitocondrias aisladas de la cepa silvestre y del mutante *Δnde1* a distintas concentraciones de NADPH. Con los datos obtenidos, que se ajustan a la cinética de Michaelis-Menten, se han podido determinar los parámetros cinéticos K_M y V_{max} de la actividad NADPH deshidrogenasa de las mitocondrias aisladas de ambas cepas. El estudio de estos parámetros cinéticos demuestra que la K_M(NADPH) es menor en el caso de las mitocondrias procedentes de la cepa silvestre que del mutante. Por tanto, Klnde1p presenta mayor afinidad que Klnde2p para el NADPH, lo que implica que la concentración de NADPH citosólico puede modular la actividad de ambas enzimas. La diferencia en la K_M de Klnde1p y Klnde2p es uno de los mecanismos que explica porque el mutante *Δklnde1rag2* no puede crecer en glucosa. Esta afirmación se ve apoyada por los fenotipos derivados de la sobreexpresión de los genes que codifican para estas proteínas en el mutante *pgi1* de *S. cerevisiae*; mientras el gen *KlNDE1* restaura el crecimiento de este mutante en medios con un 2% de glucosa, el gen *KlNDE2* sólo permite su crecimiento en medios de hasta un 0,4% de glucosa, lo que indica que cuando el flujo de glucosa a través de la ruta de las pentosas fosfato es alto, la velocidad de oxidación de NADPH a través de la proteína Klnde2p no es suficiente para evitar que la falta de NADP bloquee la ruta de las pentosas fosfato, de forma que se impidide el crecimiento de este mutante en glucosa. Además, la necesidad de la rápida reoxidación del NADPH seria todavia más acusada en *K. lactis* debido a que la actividad de esta ruta es más elevada en esta levadura que en *S. cerevisiae* (Jacoby *et al.*, 1993).

En consecuencia, dos factores explican la incapacidad del mutante *Δklnde1rag2* de crecer en glucosa: la diferente afinidad de las deshidrogenasas alternativas externas de *K. lactis* por el NADPH, siendo mayor la afinidad de Klnde1p, y la regulación positiva por glucosa que opera sobre esta proteina.

Como conclusión general de este trabajo se destaca que las deshidrogenasas alternativas externas de las mitocondrias de *K. lactis*, principalmente Klnde1p, poseen la mayor capacidad para la reoxidación del NADPH citosólico generado por el uso de la ruta de las pentosas fosfato.

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ANEXO I
MATERIALES Y MÉTODOS

1. MATERIAL BIOLÓGICO

A continuación se describen las cepas bacterianas de *Escherichia coli* empleadas en este trabajo para la amplificación de los plásmidos manejados y de las construcciones creadas, así como las cepas de levaduras utilizadas.

1.1. CEPAS BACTERIANAS

DH10B [*F' mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80dlacZΔM15 ΔlacX74 deoR recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ' rpsL nupG*]
GibcoBRL

HB101 [*supE44, hsdS20 (rB' rB') recA13, ara-14, proA2, lacY1, galK2, rpsL20, xyl-5, mtl-1*]
Bolívar y Backman, 1979

XL1-Blue [*Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac F'[proAB⁺ lac1^q lacZΔM15 Tn10(Tet')]*]
Stratagene®

DH5α [*endA1, hsdR17(r_k' m_k⁺), supE44, thi-1, recA1, gyrA(Na1⁺), relA1, Δ(lacZYA-argF) U169, (ϕ80d lacZ ΔM15)*]
Dr. Per Sunnergahen

1.2. CEPAS DE LEVADURAS

•Saccharomyces cerevisiae

GH1 [*MATα, trp1-289, leu2-3, leu2-112, gal1Δ152, lys2*]
Dr. Richard Zitomer

BY4741 [*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*]
Euroscarf

BY4742 [*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*]
Euroscarf

Y00726 [BY4741 *YMR145c::kanMX4*] YMR145c corresponde al gen *NDE1*
Euroscarf

Y12737 [BY4742 *YPL091w::kanMX4*] YPL091w corresponde al gen *GLR1*
Euroscarf

Y24190 [*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0 YDR353w::kanMX4/YDR353w*] YDR353w corresponde al gen *TRR1*
Euroscarf

Y11934 [BY4742 *YHR106w::kanMX4*] YHR106w corresponde al gen *TRR2*
Euroscarf

ENY.WA-1B [*MATa ura3-52 leu2-3,112 trp1-289 MAL2-8c MAL3 SUC3*]
Dr. E. Boles

EBY22 [ENY.WA-1B *pgi1Δ ::TRP1*]
Dr. E. Boles

•Kluyveromyces lactis

NRRL-Y1140 [*MATa*, ATCC8585, CBS2359]
Jong y Edwars, 1990

PM5-2D [*MATa, uraΔ1-1, metΔ1-1, argΔ1-1, trpΔ1-1, rag2-1*]
Dra. Micheline Wésolowski-Louvel

***rag2* ::loxP** [*MATa*, CBS2359 *rag2*::loxP]
Dr. H. Y. Steensma

PM5-3C [*MATa uraA Rag*⁺]
Dra. Micheline Wésolowski-Louvel

MW190-9B [*MATa lac4-8 uraA Rag*⁺]
Dra. Micheline Wésolowski-Louvel

2. MEDIOS DE CULTIVO

A continuación se describen los medios utilizados en cultivos de bacterias y levaduras. Para la preparación de medios sólidos se adicionó 1,5% de Bacto-Agar al correspondiente medio líquido. Las soluciones se esterilizaron en autoclave durante 20 minutos a 121°C y 2Ba

de presión. La temperatura de incubación de los cultivos fue de 37°C para bacterias y de 30°C para levaduras.

2.1. MEDIOS DE CULTIVO PARA BACTERIAS

- **LB (Luria-Bertani) modificado con glucosa**

Se trata de un medio general para crecimiento de bacterias; se compone de: 1% Bacto-Triptona, 0,5% *Bacto-Yeast-Extract*, 0,5% cloruro Sódico y 0,1% glucosa.

LB suplementado con antibiótico (LBA o LBK)

Se trata de un medio suplementado con una solución de ampicilina 40mg/mL o kanamicina 10mg/mL, para el crecimiento de bacterias transformadas con plásmidos que les proporcionan la resistencia a este antibiótico gracias a que son portadores del gen que lo permite.

La solución de ampicilina o kanamicina se prepara con agua destilada estéril y se conserva en alícuotas a una temperatura de –20°C. La solución de ampicilina se añade al medio en una proporción de 1mL por litro de medio y la de kanamicina es en una proporción de 30μL por 100mL de medio.

LBA suplementado con X-Gal+IPTG.

Medio LBA suplementado con X-Gal (5-Bromo-4-cloro-3-indolil-β-D-galactopiranósido), un substrato cromogénico. Se utilizó para discriminar entre colonias blancas y azules (productoras de β-galactosidasa), a fin de identificar los plásmidos recombinantes por la pérdida de la actividad β-galactosidasa debida a la integración de un fragmento en el sitio de clonaje múltiple (*MCS*) que interrumpe el gen *lacZ* inactivándolo.

Antes de proceder a la siembra en placa, se extendieron sobre cada una de ellas 50 μL de una solución 20 mg/mL de X-Gal en dimetilformamida y 10 μL de una solución de 0,23 mg/mL de IPTG (Isopropil β-D-tiogalactopiranósido) en agua estéril. El IPTG es un inhibidor del represor *lac* y por lo tanto inductor del gen *lacZ* que codifica para la enzima β-galactosidasa en *E. coli*.

- **SOC**

Se trata de un medio líquido empleado para la preparación de bacterias competentes. Su composición es: 2% Triptona, 0,5% Extracto de levadura, 0,06% NaCl y 0,0186% ClK.

2.1.1. Mantenimiento de las cepas de bacterias

Las cepas de bacterias se conservaron sobre placas LB.

Las cepas transformadas con plásmidos se mantuvieron en placas LBA por la resistencia conferida por el vector.

Las placas se mantuvieron a una temperatura de 4°C, realizándose resiembras periódicas cada dos meses.

El mantenimiento de las cepas a largo plazo se realiza en medio LB con un 15% de glicerol a una temperatura de –80°C, si se trata de una cepa transformada se realiza en medio LBA con un 15% de glicerol también a una temperatura de –80°C.

2.2. MEDIOS DE CULTIVO PARA LEVADURAS

- **YPD**

Consiste en un medio general para el crecimiento de levaduras. Su composición es: 1% Extracto de levadura, 2% Bacto-Peptona y 2% D-glucosa.

- **YPF**

Similar al anterior pero con 0,5% de fructosa en lugar de 0,5% de glucosa.

- **YPD anaerobio**

Medio YPD con un 0,5% de glucosa en lugar del 2% y suplementado con 0,002% ergosterol.

- **YPD geneticina**

Medio YPD suplementado con geneticina a una concentración final de 0,3 mg/mL.

- **YPEG (placas)**

Se preparó según Herrmann et al. (1994).

Se compone de extracto de levadura al 1%, bacto-peptona al 2% y agar al 2%. La mezcla anterior se ajusta a pH=5 con HCl y se esteriliza en el autoclave de modo independiente.

Cuando la solución anterior alcanza una temperatura de 65°C, se añaden glicerol al 3% v/v de un *stock* al 87% y etanol absoluto al 2% v/v.

- **CM glucosa (Medio completo)**

Se preparó según Zitomer y Hall (1976). Se trata de un medio sintético preparado de diferente modo en función de la selección que se vaya a realizar. Se compone de:

- 5 mL/L de mezcla de aminoácidos 200x cuya composición es: 2 g/L Arginina, 12 g/L Isoleucina, 8 g/L Lisina, 2 g/L Metionina, 2 g/L Treonina y 12 g/L Fenil-Alanina.
- Se añadió la siguiente mezcla de aminoácidos y bases nitrogenadas, eliminando en cada caso el utilizado para la selección o sin eliminar ninguno cuando es utilizado exclusivamente para crecimiento: 40 mg/L Histidina, 40 mg/L Adenina, 40 mg/L Leucina, 40 mg/L Tirosina, 40 mg/L Uracilo y 30 mg/L Triptófano.
- Glucosa a una concentración del 2%. Este mismo medio también se ha utilizado con otras concentraciones de glucosa: 1%, 0,5%, 0,2% ó 0,1%.
- Se ajusta el volumen con agua destilada y se esteriliza en el autoclave. Se añade YNB*, previamente esterilizado en un autoclave, a una concentración final de 0.67 % antes de verter en las placas.

*El **YNB** (*Yeast Nitrogen Base*) se compone de: 50 mL/L de Vitaminas 300x♣, 100 mL/L de Sales Traza 150x°, 15 g/L de Fosfato de Potasio, 7,5 g/L de Sulfato de Magnesio, 1,5 g/L de Cloruro Sódico, 1,5 g/L de Cloruro Cálcico y 75 g/L de Sulfato amónico.

♣ Las **Vitaminas 300x** se componen de: 0,6 mg/L Biotina, 120 mg/L Pantotenato Cálcico, 0,6 mg/L Ácido Fólico, 600 mg/L Inositol, 120 mg/L Niacina, 60 mg/L p-Aminobenzoico, 120 mg/L Piridoxina, 60 mg/L Riboflavina y 120 mg/L Tiamina.

°Las **Sales Traza 150x** se componen de: 75 g/L de Ácido Bórico, 6 mg/L de Sulfato Cúprico, 15 mg/L de Ioduro Potásico, 30 mg/L de Cloruro Férrico, 30 mg/L de Molibdato Sódico y 60 mg/L de Sulfato de Zinc.

- **CM fructosa**

Similar al anterior pero con un 2% de fructosa en lugar del 2% de glucosa, además se le añade un 0,05% de glucosa si van a cultivar las cepas PM5-2D o EBY22.

- **CM glicerol**

Similar al anterior pero con un 5% de glicerol (v/v) de un stock al 99% en lugar del 2% de glucosa y con un 0,05% de glucosa.

- **CM acetato**

Similar al anterior pero con un 5% de ácido acético absoluto (v/v) en lugar del 2% de glucosa y con un 0,05% de glucosa. Se ajusta el pH a 5,5.

- **CM etanol**

Similar al anterior pero con un 5% de etanol absoluto (v/v) en lugar del 2% de glucosa, y con un 0,05% de glucosa.

- **CM galactosa**

Similar al anterior pero con un 2% o 0,5% de galactosa en lugar del 2% de glucosa.

- **CM lactato**

Similar al anterior pero con un 2,2% de lactato (v/v) de un *stock* al 90% en lugar del 2% de glucosa y con un 0,05% de glucosa. La solución es ajustada a pH 5,5 con KOH 10M antes de ser esterilizada en el autoclave.

Cuando usamos el medio con lactato para la inducción de formación de mitocondrias, la composición es la siguiente: extracto de levadura al 0,3%, glucosa al 0,091%, KH_2PO_4 al 0,1%, NH_4Cl al 0,1%, $CaCl_2{*}2H_2O$ al 0,05%, NaCl al 0,05%, $MgSO_4{*}7H_2O$ al 0,11%, $FeCl_3$ al 0,03% (v/v) de un *stock* al 1% y lactato al 2,2% (v/v) de un *stock* al 90%. La solución es ajustada a pH 5,5 con KOH 10M antes de ser esterilizada en el autoclave.

- **CM-ura**

Similar al CM glucosa pero en ausencia de uracilo.

- **CM-ura geneticina**

Similar al CM glucosa pero en ausencia de uracilo y suplementado con geneticina a una concentración final de 0,3 mg/mL.

- **Placas CM, CM-ura y YPD antimicina A**

Placas del medio específico sólido suplementadas con antimicina A a una concentración final de 5μM.

- **MM-glucosa (Medio mínimo)**

Similar al CM-glucosa pero sin añadir ningún aminoácido o base nitrogenada, salvo los necesarios para que pueda crecer la cepa en cuestión, es decir, los aminoácidos para los cuales la cepa es auxotrófica. Estos aminoácidos se añaden a una concentración de 40mg/L.

- **MM-fructosa**

Similar al MM-glucosa pero en lugar de añadir glucosa al 2% se añade fructosa al 2%, y si se va a utilizar con la cepa PM5-2D también se le añade glucosa al 0,1%.

- **CM para la cepa PM5-2D**

Si se van a utilizar con esta cepa tanto el medio CM-glucosa como el medio CM-fructosa, éstos deben ser suplementados con los aminoácidos arginina y metionina a una concentración de 40mg/L.

2.2.1. Mantenimiento de las cepas de levadura

Las cepas de levadura se conservaron habitualmente sembradas en placas YPD.

Las placas se conservaron a 4° C realizándose resiembras periódicas cada dos meses.

Para conservar las cepas de levadura durante un largo período de tiempo se congelan y se mantienen a una temperatura de –80°C en el medio de cultivo de elección con un 50% de glicerol.

3. VECTORES DE CLONACIÓN, MARCADORES MOLECULARES Y GENOTECA

3.1. VECTORES DE CLONACIÓN

pGEM®- T Easy [*ori f1ori amp*^{r} *lacZ T7/SP6 RNA pol. promoters*] (PROMEGA Co. Madison WI) Fue utilizado para clonar productos de PCR.

YEplac195 (Gietz y Sugino, 1988). Presenta el promotor y 438 pb del gen de la β-galactosidasa de *E. coli* (*lacZ*) (Sambrook *et al.*, 1989), *amp*^{r}, *URA3*, 2μm de la levadura y el *ori* bacteriano. Es un vector lanzadera que frecuentemente se emplea para la construcción de genotecas. En el presente trabajo se empleó para la realización de ensayos de complementación.

pXW2 (Chen *et al.*, 1992). Plásmido lanzadera empleado para el análisis de promotores fusionados al gen *lacZ* de la β-galactosidasa de *E. coli*. Presenta la *amp*^{r}, el *ori* bacteriano, el *S11* para la replicación en *K. lactis*, el marcador de selección *URA3*, y los genes *lacY*, *lacA* y *lacZ* que codifican para una permeasa, una tiogalactósido transacetilasa y para la β-galactosidasa de *E. coli*.

pET21d(+) (*Novagen*). Este vector se ha empleado para la expresión de proteínas en *E. coli*. El gen deseado se clona bajo el control del promotor T7, el cual no es reconocido por la ARN polimerasa de *E. coli* y, por tanto, la expresión no tiene lugar hasta que se le proporcione una fuente de T7 ARN polimerasa. Una vez que el plásmido es transferido a un hospedador de expresión, conteniendo una copia cromosomal del gen T7 ARN polimerasa bajo el control de *lacUV5*, la expresión de la proteína es inducida por la adición de IPTG (Isopropil β-D-tiogalactopiranósido).

pYES2-GFP (Dr. Markus J. Tamás). Este vector se ha usado para estudios de localización subcelular, siendo un plásmido episómico dónde se inserta la secuencia codificadora de la proteína que queremos estudiar fusionada al gen *GFP* que codifica para la proteína fluorescente verde, bajo el control del promotor *GAL1* y el terminador *CYC1*. Poseen origen de replicación para *E. coli* y para *S. cerevisiae*, el gen de resistencia a la Ampicilina y el gen *URA3* que codifica para una enzima que participa en la síntesis de uracilo.

3.2. MARCADORES MOLECULARES

Para calcular los tamaños de los fragmentos de DNA digeridos y de los productos de PCR empleamos como marcadores el DNA del fago lambda digerido con la enzima de restricción *Bst*EII (Daniels et al., 1983), así como el *MarkerII* de *Roche* y el de 500pb de BSA que se puede ver en la figura 2. También se ha empleado el *MarkerII* de 125 a 23130 pb de *Roche* que procede de la digestión del bacteriófago lambda con la endonucleasa de restricción *Hind*III, pero marcado con digoxigenina, en experimentos de *Southern blot*.

Se empleó el marcador de proteínas *Prestained SDS-PAGE Standards Broad Range* de *Bio-Rad*, que comprende desde 6,4 a 198 kDa (figura 2), como referencia de tamaños en electroforesis de proteínas en geles de poliacrilamida (*SDS-PAGE*).

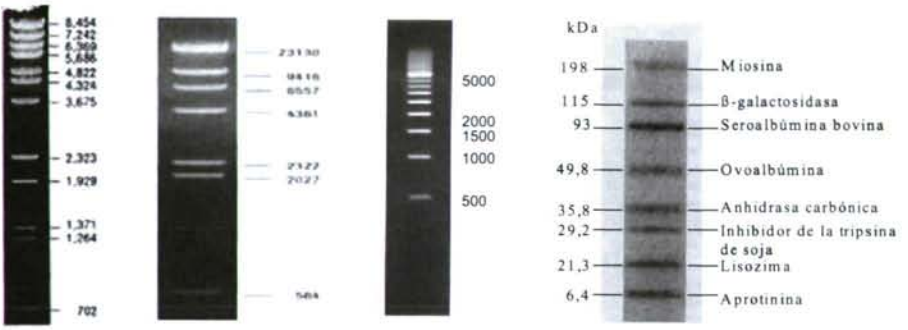

Figura 1: De izquierda a derecha: marcador molecular *Lambda*, marcador molecular *Marker II*, marcador de 500pb de BSA y marcador de peso molecular de proteínas.

3.3. GENOTECA DE *K.lactis* EN EL VECTOR KEp6

Para la técnica de selección de clones por DCbyPCR, se empleó una genoteca de *K. lactis* insertada en el sitio *Bam*HI de clonación del vector KEp6 (figura 1).

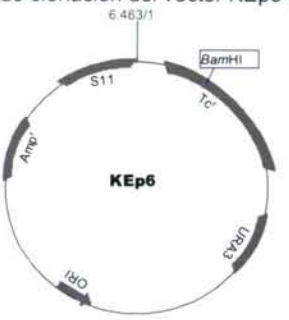

Figura 2: Vector KEp6 con el origen de replicación S11, que es específico para *K.lactis*.

4. TÉCNICAS DE BIOLOGÍA MOLECULAR

4.1. OBTENCIÓN DE ÁCIDOS NUCLEICOS

4.1.1. Obtención de DNA plasmídico a partir de bacterias

El aislamiento de los plásmidos se realizó según el método de lisis alcalina (Sambrook et al., 1989), extracción con fenol y precipitación del DNA con etanol al 95% a –20°C.

Para realizar el aislamiento a pequeña escala (miniprep) se sembraron en estría las colonias de bacterias en placas LBA. Tras su crecimiento durante toda la noche a 37°C, las células se recogieron con la ayuda de un palillo y se resuspendieron en 100µL de solución I (glucosa 50mM, EDTA 10mM, Tris-ClH 25mM, pH 8,0) previamente almacenada en la nevera. Posteriormente se añadieron 200µL de la solución II preparada en el momento (NaOH 0,2N y SDS al 1%), se mezcló por inversión 2 ó 3 veces hasta conseguir una viscosidad homogénea; después se añadieron 200µL de la solución III (60mL de acetato potásico 5M, 11,5mL de ácido acético glacial y 28,5 mL de agua destilada) que se mezcló por inversión suave del tubo. Los tubos se centrifugaron a 12.000r.p.m. durante 5 minutos. Para eliminar restos de proteínas que podrían dificultar posteriores manipulaciones del DNA, el sobrenadante se sometió a una extracción con igual volumen de PCIA*, se agitó la mezcla en vortex y se centrifugó 5 minutos a 8.000r.p.m. Se transfirió la fase superior acuosa a un tubo limpio y se le añadieron 2 volúmenes de etanol al 95%, se incubó 10 minutos a –20°C y, posteriormente, los plásmidos se precipitaron por centrifugación a 13.000r.p.m. durante 15 minutos. Para eliminar restos de sales, el sedimento se lavó con un volumen de etanol al 70% y se precipitó por centrifugación a 13.000r.p.m. durante 5 minutos. Las muestras se secaron al vacío por espacio de 5 minutos y posteriormente se resuspendieron en 50µL de agua destilada estéril. Las preparaciones se conservaron congeladas a –20°C hasta su utilización.

En las preparaciones a mediana escala (maxiprep) se procedió de igual modo que el mencionado en este apartado, pero partiendo de mayor cantidad de colonias de células y variando el volumen de las soluciones en la proporción correspondiente.

***PCIA** (Fenol: cloroformo: alcohol isoamílico)

Se mezclaron un volumen de fenol fundido a 65°C con un volumen de cloroformo y con dos volúmenes de tampón 1×TE(Tris-ClH 0,1M, LiCl 0,1M, EDTA 0,1mM pH 7,5) para equilibrar el pH. Se agitó vigorosamente durante varios minutos, tras los cuales se dejó la mezcla a temperatura ambiente y en oscuridad hasta que se separó completamente la fase acuosa que se retiró por aspiración. Se repitió este proceso dos o tres veces. Tras el último equilibrado se añadieron 1/25 volúmenes de alcohol isoamílico a la mezcla. Se conservó en oscuridad a 4°C.

♣Tratamiento con RNAsa A

Con el procedimiento anterior, además de DNA, se obtiene una considerable cantidad de RNA, así que para las reacciones de PCR de reconocimiento de clones y para las digestiones, el DNA empleado como molde fue tratado con 0,1µg de RNAsa por cada mL de DNA. Se dejó actuar la enzima durante 15 min a 37° C y luego se utilizó la mezcla para la reacción.

Para la preparación de la RNAsa a una concentración de 6 mg/mL se utilizó la Ribonucleasa A de páncreas bovino de Sigma® como se describe a continuación. Se pesaron 18 mg de Ribonucleasa A y se disolvieron en 3 mL de tampón Tris-ClH 10 mM, pH 8.5. Seguidamente, se mantuvo la mezcla durante 18 min a 100° C para permitir la desnaturalización de las DNAsas (las RNAsas son más resistentes a las altas temperaturas), y luego se dejó enfriar a temperatura ambiente. Se almacenó en alícuotas a –20° C.

4.1.2. Extracción mediante columnas de QIAprep®

Las células se inocularon en 5 mL de medio LB suplementado con el antibiótico adecuado y se dejaron crecer toda la noche en agitación a 37° C. El cultivo se repartió en tubos eppendorff. Se centrifugó durante 2 minutos a 4.000 r.p.m. y se eliminó el sobrenadante. Las células se resuspendieron en 250 µL de tampón P1 (se utilizaron los reactivos suministrados por el *kit*) agitando en el vortex y posteriormente se añadieron 250 µL de tampón P2. Se mezcló el tubo por inversión y se añadieron 350 µL de tampón N3 para agitar nuevamente en el vortex. Seguidamente se sedimentaron las células durante 10 minutos a 13.000 r.p.m. El sobrenadante fue transferido a la columna del kit para centrifugar a 13.000 r. p. m. durante 1 minuto. Se eliminó el líquido residual, y se lavó la columna con 1 mL de tampón PB. Tras una centrifugación de 1 minuto a 13.000 r.p.m. y eliminación de nuevo del líquido restante, se añadieron 750 µL de tampón PE. Sigue otra centrifugación de 1 minuto a 13.000 r.p.m., eliminación del tampón residual y nueva centrifugación de 1 minuto a 13.000 r.p.m. para descartar los restos de tampón. Las columnas se colocaron sobre un eppendorff limpio y se añadieron 50 µL de agua. Tras 2 minuto de espera, se centrifugó 1 minuto a 13.000 r.p.m. y se recogió la suspensión de DNA.

4.1.3. Extracción de DNA de alto peso molecular de levaduras

Las extracciones se realizaron partiendo de cultivos de 10 mL con una $D.O._{600} \approx 0.6$, siguiendo el procedimiento que se describe a continuación:

Las células se centrifugaron a 5.000 r.p.m. durante 3 minutos y se lavaron con 10 mL de Sorbitol 1 M suplementado con EDTA 0.1 M. Se centrifugaron de nuevo a 5.000 r.p.m. durante 5 minutos y se resuspendió el *pellet* en 1 mL de la solución de extracción (Sorbitol 0,9 M, EDTA 50 mM, Na_2PO_4 50 mM pH 7,5, 14 mM β-mercaptoetanol). A la mezcla se le añadieron 0,05 mL de liticasa, previamente disuelta a una concentración de 5 mg/mL en agua estéril y se agitó suavemente durante 30 minutos a 30° C. Tras este período de incubación, se añadió SDS hasta una concentración final del 1% y se mezcló suavemente. Se incubó durante 20 minutos a 30° C sin agitación y se hizo un lavado con 1,5 veces el volumen de PCIA, agitando suavemente, para evitar roturas en el DNA. Las fases se separaron por centrifugación a 5.000 r.p.m. durante 10 minutos para conseguir una fase acuosa lo más limpia de proteínas posible. Para completar la extracción de proteínas, la fase acuosa se incubó durante 2-4 horas en hielo con acetato potásico 5 M (pH 4,8) en una proporción 1:5, tras lo cual se centrifugó 15 minutos a 10.000 r.p.m. Se recogió el sobrenadante, descartando así el posible precipitado que se produce cuando quedan restos de proteína. Una vez purificado, el DNA genómico se precipitó con 1,5 volúmenes de etanol al 95%, mezclándolo suavemente. Se dejó en hielo unas dos horas y transcurrido este tiempo se centrifugó a 8.000 r.p.m. durante 5 minutos. El DNA se lavó con etanol al 70% para eliminar los restos de sales y tras una nueva centrifugación de 2 minutos a 5.000 r.p.m. se secó el precipitado a vacío y se resuspendió en agua estéril.

4.1.4. Extracción rápida de DNA genómico de levaduras

Se empleó el método de Hoffman y Winston (1987) con ligeras modificaciones cuando fue necesario obtener pequeñas concentraciones de ADN genómico en el menor tiempo posible, para ello se partió de un cultivo de 10 mL de levaduras crecido durante una noche. Se centrifugó 5 minutos a 4.000 r.p.m. y se eliminó el sobrenadante. Las células se resuspendieron en 500 µL de agua y se transfirieron a un tubo *Eppendorff*. Se centrifugaron durante 10 segundos a 13.000 r.p.m., se eliminó el sobrenadante y se rompieron las células agitando en *vortex* brevemente. Para ello se resuspendieron las células en 200 µL de tampón *Breaking* (2% v/v Tritón X-100, 1% v/v SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8, 1mM EDTA pH 8) y 3 µL de *RNAsa* 50 mg/mL (suministrada por *Quiagen*). Se añadieron 0,3 gramos de perlas de vidrio, 200 µL de fenol-cloroformo y se agitó en *vortex* durante 3 minutos a baja velocidad. Se añadieron 200 µL de tampón 1xTE (10 mM Tris-HCl pH 7,5 y 1 mM EDTA) y se agitó en *vortex* brevemente. Se centrifugó 5 minutos a 13.000 r.p.m. a temperatura ambiente, y se transfirió el sobrenadante a un tubo *Eppendorff* limpio. Después de añadir 1 mL de etanol al 100% se mezcló por inversión y se centrifugó durante 3 minutos a 13.000 r.p.m. a temperatura ambiente. Se eliminó el sobrenadante, se secó el ADN en una bomba de vacío y se resuspendió en 20-100 µLde 1xTE.

4.1.5. Extracción de plásmido de levadura empleando perlas de vidrio

Este método se empleó para obtener pequeñas cantidades de ADN plasmídico, para ello se partió de un cultivo de 10 mL de levaduras transformadas creciendo en medio selectivo. Se centrifugaron las células a 4.000 r.p.m. durante 3 minutos, el *pellet* se resuspendió en 200µL de la solución I (glucosa 50mM, EDTA 10mM, Tris-ClH 25mM, pH 8,0). Se añadieron perlas de vidrio de 0,4mm de diámetro hasta justo debajo del nivel del líquido. Se agitaron en *vortex* a máxima velocidad durante 4 minutos y se centrifugaron a 13000 r.p.m. durante 2 minutos. Se recogió el sobrenadante en un tubo *Eppendorff* limpio y se añadieron 200µL de la solución II preparada en el momento (NaOH 0,2N y SDS al 1%), se mezcló por inversión 2 ó 3 veces hasta conseguir una viscosidad homogénea; después se añadieron 150µL de la solución III (60mL de acetato potásico 5M, 11,5mL de ácido acético glacial y 28,5mL de agua destilada) y se incubó la suspensión en hielo durante 5 minutos. Se añadió un volumen de PCIA (descrito anteriormente) y se agitó en *vortex* a máxima velocidad brevemente. Posteriormente se centrifugó a 13000 r.p.m. durante 2 minutos, se recogió el sobrenadante a un tubo limpio y se precipitó con 2 volumenes de etanol al 95% más 0,1 volumen de acetato de sodio 3M durante 30 minutos en hielo. Se centrifugó la mezcla a 13000 r.p.m. durante 10 minutos, el precipitado se lavó con etanol al 70% y finalmente se secó en una bomba de vacío. El DNA plasmídico se resuspendió en 5 µL de agua destilada y fue empleado para transformar bacterias de *E. coli* por el método del cloruro de rubidio.

4.1.6. Extracción de RNA total de levaduras

Para la extracción de RNA total de levaduras, al igual que en todos aquellos procesos en los que se llevó a cabo la manipulación de RNA y con el fin de evitar posibles contaminaciones de RNAsas, todo el material utilizado, tanto plástico como cristal, fue utilizado en condiciones estériles y previamente lavado con agua bidestilada tratada con dietilpirocarbonato (DEPC) al 1% durante 24 horas, y esterilizada en autoclave para inactivar totalmente el DEPC. Todas las disoluciones fueron preparadas con agua tratada.

Se partió de cultivos de 200mL, en distintas condiciones dependiendo del experimento en concreto. Una vez alcanzada una densidad óptica cercana a 0,6, los cultivos se enfriaron rápidamente en hielo, dejándolos unos 15 minutos en hielo para impedir, en lo posible, la degradación del RNA y asegurar el cese del crecimiento y de la transcripción. Todos los procesos posteriores se realizan en frío a 4ºC.

Las células se centrifugaron a 5.000r.p.m. durante 5 minutos y se lavaron con 20mL de 1×RE (Tris-ClH 0,1M, LiCl 0,1M, EDTA 0,1mM pH 7,5). En este punto, el sedimento se resuspendió en 2mL de 1×RE y fue utilizado inmediatamente para la extracción o congelado a –20ºC hasta su utilización posterior.

El sedimento celular se resuspendió en 1,5mL de 1×RE y se le añadieron perlas de vidrio, de 1mm de diámetro, hasta 2/3 del volumen final. Se realizaron pulsos de agitación vigorosa en vórtex de 20 segundos, y 20 segundos de incubación en hielo para impedir que la muestra se calentase por la fricción. Se repitió esto 9 veces, tras lo cual se añadieron 2 mL de 1×RE con un 1% de SDS. Se mezcló bien y se añadieron 7,8 mL de PCIA saturado con 1×RE para proceder a la extracción de proteínas, repitiendo este paso hasta que la fase acuosa perdió la turbidez provocada por el SDS, agitando unos 20 segundos en el vórtex y centrifugando 5 minutos a 10.000r.p.m. para separar las fases. La fase acuosa se centrifugó

con 2,3 volumenes de etanol al 95% y 1/10 del volumen de acetato sódico 3M, incubando unas horas a –20°C.

El RNA precipitado se centrifugó a 10.000r.p.m. durante 10 minutos y se lavó 2 veces con etanol al 75%, tras lo cual se secó al vacío, se resuspendió en agua tratada y se almacenó a –80°C hasta su posterior utilización.

4.2. MEDIDA DE LA CONCENTRACIÓN DE ÁCIDOS NUCLEICOS

4.2.1. Medida de la concentración de DNA

Se tomaron 5μL de la muestra de DNA a medir, libre de RNA, y se llevaron a un volumen final de 1mL con H_2O bidestilada estéril. Las medidas se realizaron en un espectrofotómetro en cubetas de cuarzo de 1mL. La absorbancia se midió a dos longitudes de onda, 260 y 280nm, y la concentración se calculó sabiendo que una unidad de absorbancia a 260nm indica una concentración de 50μg/mL de DNA de doble hebra.

La relación A_{260}/A_{280} es un índice del grado de impurezas que puede haber en la muestra. La relación ideal se ajusta a un valor aproximado de 1,8 (Sambrook *et al.*, 1989).

La concentración de aquellas muestras a las que no se les realizó una extracción de RNA fue estimada en geles de agarosa teñidos con bromuro de etidio, comparando la intensidad de fluorescencia de las bandas de las muestras con un marcador de concentración conocida, mediante el programa informático Quantity One® versión 4.5.0 de la casa comercial Bio Labs.

4.2.2. Medida de la concentración de RNA

Las medidas de RNA se realizaron del mismo modo que las de DNA, utilizando 4μL de muestra llevados a 1mL en H_2O tratada con DEPC, teniendo en cuenta que una unidad de absorbancia a 260nm equivale a 40μg/mL de RNA y que la relación óptima A_{260}/A_{280} es de 2 (Sambrook et al., 1989).

4.3. MODIFICACIÓN ENZIMÁTICA DEL DNA

4.3.1. Digestión del DNA con endonucleasas de restricción

Tanto el DNA plasmídico como el genómico fueron digeridos con endonucleasas de restricción de diversas casas comerciales siguiendo sus recomendaciones en cuanto al tampón y a la temperatura utilizadas. Los tiempos de digestión variaron entre las 2 y 4 horas según la cantidad de DNA a digerir y las enzimas empleadas.

4.3.2. Ligamiento de fragmentos de DNA

• Ligamiento mediante el *kit pGEM®- T Easy Vector System I*

Para la unión al vector p-GEM®-T Easy se realizaron reacciones de ligamiento empleando el *kit pGEM®- T Easy Vector System I* de *Promega* siguiendo sus indicaciones:

8 μL del inserto rescatado y concentrado
1 μL de Ligasa T4 (3U/μL)
10 μL de Rapid Buffer 2x
1 μL de vector pGEM-T-Easy diluido 1/10 (50ng)

Se dejó la reacción durante una noche a 14° C para luego transformar con todo el volumen bacterias competentes de la línea DH10B y seleccionar en placas LBA.

• Ligamiento mediante DNA ligasa T_4

Se emplearon la DNA ligasa T_4 de *Roche* o de *GibcoBRL* con el tampón suministrado con la enzima. Los ligamientos se realizaron en un volumen de 10 μL añadiendo un volumen variable de DNA (generalmente una proporción 1:10 de vector e inserto a clonar) y 1 U de ligasa. Se incubó 2-4 horas a temperatura ambiente o toda la noche a 4°C cuando el ligamiento era de extremos cohesivos. Cuando el ligamiento era de extremos romos se incubó a 14-18°C toda la noche.

• Ligamiento mediante la técnica *GAP-repair*

Está basada en los mecanismos de reparación de mellas en el DNA por recombinación.

Se genera una mella en la región del plásmido dónde se quiere insertar nuestro fragmento de DNA con una o dos enzimas de restricción y se amplifica por PCR el fragmento que se quiere insertar con cebadores construidos portando colas de 30 nucleótidos homólogos a la región del plásmido dónde se quiere insertar la secuencia.

Se cotransforma *S. cerevisiae* con el producto de PCR y el plásmido lineal, y la levadura realiza la recombinación generando un plásmido circular que contiene nuestro fragmento de DNA en la posición que nosotros hemos elegido.

La modificación de este método de Zaragoza (2003) se ha usado para la construcción de secuencias para la disrupción de genes.

4.3.3. Amplificación del DNA mediante la reacción en cadena de la polimerasa (PCR)

El principio de la PCR se basa en utilizar de manera repetitiva una de las propiedades de las DNA polimerasas: la capacidad de sintetizar una hebra complementaria de DNA a partir de un iniciador o DNA cebador y un molde de hebra simple. En general se utiliza la Taq DNA polimerasa obtenida de una bacteria de aguas termales (*Thermus aquaticus*), que tiene su temperatura óptima en 72ºC, y es estable a los 94ºC necesarios para realizar la desnaturalización. La utilización de esta enzima ha permitido la automatización de la PCR.

Los productos empleados son los siguientes:

Mezcla de PCR:

17,55µL de H_2O destilada

2,5µL de tampón de la Taq Polimerasa 5x

0,7µL de mezcla de dNTPs 2,5 mM

1µL de DNA molde (las extracciones de DNA de cada caso)

1,5µL de cada cebador 20pmol/µL

0,25 µL de Taq DNA polimerasa de *Roche* (5U/µL)

Los ciclos de reacción empleados en todas las reacciones de PCR de este trabajo han sido iguales. Tan solo variaba la temperatura de *anneling* que dependía de la temperatura de *melting* de los cebadores.

Ciclos de PCR:

1.....ciclo.....	94º C....2 minutos.....desnaturalización inicial
30.....ciclos.....	94º C....30 segundos.....desnaturalización
	50º C....1 minuto.....*anneling*
	72º C....2 minutos.....elongación
1.....ciclo.....	72º C....2 minutos.....elongación final
	14º C....mantenimiento

Purificación del DNA amplificado por *PCR*

El DNA amplificado mediante la *PCR* fue purificado antes de ser utilizado para otras aplicaciones. Esta purificación permitió no solo la concentración de la muestra sino también la eliminación de los cebadores y los dNTPs no incorporados. Para ello se utilizó el *kit* comercial *Microcon-PCR* de *Millipore*.

La muestra se llevó con agua o tampón TE (10 mM Tris-HCl pH 7,5 y 1 mM EDTA) a un volumen final máximo de 0,5 mL. Se introdujo en el depósito de la columna (sin tocar la membrana con la punta de la micropipeta) y se centrifugó a 4.000 r.p.m. durante 15 minutos. A continuación se retiró el filtrado, se añadieron 20 µL de agua destilada o tampón TE muy cuidadosamente, se invirtió el depósito en donde habíamos introducido la muestra y se colocó en un tubo *Eppendorff* limpio que se centrifugó a 4.000 r.p.m. durante 2 minutos. El sobrenadante obtenido contenía el DNA purificado.

4.3.4. Preparación de sondas radiactivas de DNA.

♦SONDA ESPECÍFICA

El DNA molde se obtuvo a partir de DNA genómico de levadura por amplificación con cebadores específicos del fragmento de DNA elegido. El producto de esta PCR se separó en un gel de agarosa y se rescató y se concentró el fragmento de interés.

Las sondas se marcaron utilizando el *kit Prime a Gene System* (*Promega*). Para ello, se utilizaron 14µL de DNA molde que se desnaturalizaron a 95ºC durante 5 minutos y se enfriaron rápidamente en hielo. Al DNA molde desnaturalizado se le añadieron 7µL de la mezcla de componentes del kit. Posteriormente se añadieron 3µL de α^{32}P dATP (*Amersham*) y 1U de fragmento Klenow de la DNA polimerasa I de *E.coli*. Se incubó durante 1 hora a 37ºC y, transcurrido ese tiempo, la sonda marcada se hizo pasar por una columna de Sephadex-G50 equilibrada con STE (Tris-ClH 10mM pH 7,5, EDTA 0,1M, SDS1%), recogiendo fracciones de 200µL en tubos Eppendorff. La radiactividad incorporada en dichas fracciones fue determinada en un contador de centelleo (*Beta-Wallac*) y se recogieron aquellas muestras con la mayor carga de radiactividad.

♦SONDA DE RNA RIBOSÓMICO 25s

La sonda se realizó con 1µL de oligonucleótido de rRNA 25s que se encuentra a una concentración de 10pmol/µL suministrado por Roche Diagnostics, al cual se le añadieron: 1µL de tampón 10x para la T4 polinucleótidoquinasa, 9,5 µL de agua, 2µL de γ^{32}P dATP

(Amersham) y 1U de T4 polinucleótidoquinasa. Se dejó transcurrir la reacción durante una hora a 37°C. Esta sonda no precisa purificación.

4.3.5. Preparación de sondas de DNA marcadas con digoxigenina

Las sondas se marcaron por el método del "*random-primed*" (cebadores aleatorios) (Feinberg y Vogelstein, 1983) utilizando los reactivos suministrados en el kit *Dig DNA Labeling and Detection* (*Roche*). Para ello, se disolvieron entre 0,5 y 3 μg de ADN en 15 μL de agua, se hirvieron durante 10 minutos a 94°C para su desnaturalización, y se enfriaron rápidamente en hielo o nieve carbónica. Al DNA desnaturalizado se le añadió:

Mezcla de hexanucleótidos 10x	2 μL
Mezcla de dNTPs 10x (1mM dATP, 1 mM dCTP, 1 mM dGTP, 0,65 mM dTTP, 0,35 mM DIG-dUTP pH 7,5)	2 μL
Klenow 2U/μL	1 μL
H_2O hasta V_f	25 μL

Se mezcló bien y se incubó durante un mínimo de 1 hora a 37°C. Una vez transcurrido este tiempo, se paró la reacción añadiendo 2 μL de EDTA 0,2 M pH 8.0. Posteriormente se precipitó el DNA añadiendo 2,5 μL de LiCl 4 M , 75 μL de etanol al 95%, dejándolo durante 30 minutos a –70°C o durante 2 horas a –20°C y centrifugándolo en una microcentrífuga durante 15 minutos. A continuación se lavó el *pellet* con 50 μL de etanol al 70%, se secó a vacío y se disolvió en 50 μL de 1xTE (Tris-HCl 1 M pH 7,5, EDTA 0,5 M pH 8,0).

Para comprobar si el marcaje de la sonda había sido correcto se realizó un *Dot-blot*. Esta técnica consistió en dispensar a modo de punto, sobre una membrana de nylon cargada positivamente (*Roche*), 1 μL de varias soluciones control; una solución que contiene un DNA no marcado con la digoxigenina (control -), una solución que contiene un DNA marcado con la digoxigenina (control +) y nuestra sonda recién marcada. Generalmente se suelen hacer varias diluciones de los controles y de nuestra sonda. Los controles los suministra el kit. Después de secar la membrana en la estufa a 37°C durante 10-15 minutos se llevó a cabo la detección inmunológica (descrita en la técnica *Southern blotting*).

4.4. MÉTODOS DE TRANSFORMACIÓN DE BACTERIAS

4.4.1. Método de transformación con cloruro de rubidio (Kushner, 1978)

Preparación de bacterias competentes

Se inoculó medio SOC (ver medios de cultivo) añadiendo 1 mL de preinóculo por cada 100 mL del volumen del medio. Se mide la $D.O._{600}$ en el espectrofotómetro.

Se incubó el cultivo a 37° C y agitación (300 r.p.m.) hasta alcanzar la densidad óptica deseada ($D.O._{600} \approx 0,5\text{-}0,6$). En ese momento se introdujo el cultivo en hielo durante 10 minutos para detener la división celular. Transcurrido ese tiempo se vertió el cultivo en tubos de centrífuga estériles y previamente enfriados y se realizó una centrifugación durante 7 minutos a 5.000 r.p.m. a 4°C. Es importante que en todo momento las células se encuentren en un medio frío, por lo que a partir de este momento los tubos permanecieron en un recipiente con hielo.

Descartado el sobrenadante, se añadió una pequeña cantidad de solución TFB-1* en cada tubo de centrífuga. Se resuspendieron las células hasta que no se observó ningún grumo y se completó el volumen de TFB-1 hasta los 40 mL. Se mantuvo la suspensión en hielo durante 5 minutos para permitir la actuación del TFB-1. Transcurrido este tiempo, se centrifugó a 5.000 r.p.m. durante 7 min. Se descartó el medio dejándose únicamente las células y se añadió una pequeña cantidad de TFB-2** (también previamente enfriado). Se resuspendieron las células hasta que no quedaron grumos y se completó el volumen con el TFB-2 restante.

Finalmente se repartió la mezcla celular en alícuotas de 100 μL en tubos *Eppendorff* (previamente enfriados) que se mantuvieron en hielo seco hasta su almacenamiento definitivo a –80° C.

* 80 mL de **TBF-1** compuesto por: Cl_2Rb 0,964 g, Cl_2Mn 0,788 g, CH_3COOK 0,232 g (pH 5.8), Cl_2Ca 0,116 g y Glicerol 12 mL. Tras enrasar a 80 mL con agua bidestilada, se esterilizó en el autoclave.

** 15 mL de **TBF-2** compuesto por: Cl_2Rb 0,02 g, Cl_2Ca 0,2 g y Glicerol 2,28 mL. Tras enrasar a 15 mL con agua bidestilada, se esterilizó en el autoclave.

Transformación de las células.

La transformación se llevó a cabo utilizando alícuotas de 100μL de células competentes recién descongeladas en hielo (aproximadamente durante 15 minutos) a las que se añadió 10μL de DNA plasmídico y se incubaron a 0°C durante 30 min. Transcurrido este tiempo, se sometieron a un choque térmico a 42° C durante 1 minuto y medio para luego

añadirles 400µL de medio LB e incubarlas a 37° C durante 1 hora. Pasado este tiempo, las células se centrifugaron a 6.000 r.p.m. durante 3 minutos, se eliminó el sobrenadante y se resuspendieron en el volumen necesario de LB para sembrar finalmente 100µL de esta suspensión por placa de medio selectivo.

4.4.2. Método de transformación por electroporación

Preparación de células electro-competentes.

Se inocularon 10 mL de medio LB con las células bacterianas y se dejó crecer en agitación a 37° C durante una noche. Al día siguiente se inoculó 1 litro de LB con ese pre-inóculo y se incubó en agitación hasta alcanzar una $D.O._{600} \approx 0,5-0,6$ de modo que las células estuviesen en la fase logarítmica de crecimiento.

En ese momento, se introdujo el cultivo durante 10 min en un recipiente con hielo para detener el crecimiento. Transcurrido este tiempo, se centrifugaron las células durante 5 min a 5.000 r.p.m. para lavarlas a continuación con 50 mL de agua estéril fría. Se centrifugaron de nuevo y se volvieron a lavar de modo que se eliminen todos los restos de medio de cultivo o sales que pudieran causar descargas eléctricas y provocar la muerte de las células.

Tras dos nuevos lavados, se resuspendieron en glicerol al 10% frío.

Finalmente se volvieron a centrifugar para resuspender por último en 2 mL de glicerol al 10%. Esta suspensión celular fue repartida en alícuotas de 50 µL en tubos eppendorff previamente enfriados y que se mantuvieron en hielo seco hasta su almacenamiento definitivo a –80° C.

Transformación de las células electro-competentes

La transformación se llevó a cabo tomando una alícuota de 50 µL, recién descongelada en hielo, a la que se le añadió 1 µL de la suspensión de DNA transformante. Se mezcló suavemente y se introdujeron las células en una cubeta especial para el electroporador (*Electroporation Cuvette 1mm. EQUIBIO*). La cubeta se introdujo en el electroporador *GENE PULSER®* de *BIORAD* y se sometieron a las células a las siguientes condiciones: voltaje: 2,5 KV, capacitancia: 25 µF, resistencia: 200 Ω y tiempo: 4,8 milisegundos. Tras lo cual se añadió 1 mL de LB y se incubaron durante 30 min a 37°C. Transcurrida la incubación, se centrifugaron las células a 6.000 r.p.m. durante 10 min se eliminó el sobrenadante, se resupendieron en el volumen deseado de LB (100 µL por placa de siembra) y se sembraron en placas selectivas LBA.

4.5. REPLICACIÓN DE PLACAS (*Replica-plating*)

Se utilizó el método del terciopelo para obtener copias de las placas con alta densidad de colonias aisladas.

4.6. MÉTODOS DE TRANSFORMACIÓN DE LEVADURAS

4.6.1. Método de alta eficiencia para la transformación de *K. lactis*

Las células competentes de *K. lactis* se prepararon según Gietz y Woods (1994). Se inocularon 5 mL de medio de cultivo YPD al 0,5% para iniciar un precultivo a partir de una colonia aislada y se incubó toda la noche a 30°C en agitación. Al día siguiente se determinó la densidad óptica a 600 nm del cultivo y, a partir de él, se inoculó un nuevo cultivo de tal forma que su densidad óptica a 600 nm fuese de 0,1. Cuando el cultivo llegó a una densidad óptica de 0,6, después de aproximadamente 6 horas (aunque puede variar dependiendo de la cepa de *K. lactis* que se utilice), se centrifugaron las células a 3.500 r.p.m. durante 5 minutos. El sedimento se resuspendió en 1mL de agua destilada estéril y se transfirió a un tubo *Eppendorff* estéril. Las células se volvieron a centrifugar y se resuspendieron en acetato de litio 100 mM. Una vez resuspendidas, las células fueron incubadas a 30°C durante 30 minutos sin agitación.

Tras la preparación de las células competentes, se llevó a cabo la transformación añadiendo 5 µL de DNA de salmón (10 mg/mL), previamente desnaturalizado durante 10 minutos a 95°C, y 1-5 µg de DNA plasmídico, a cada tubo *Eppendorff* que contenía 50 µL de células competentes. Se mezcló bien y se incubó a 30°C durante 30 minutos sin agitación. Transcurrido este tiempo, se añadieron 300 µL de una disolución de acetato de litio 100 mM y PEG-3.350 al 40%, preparada en el momento a partir de acetato de litio 1M y PEG-3.350 al 50% (p/v). Se mezcló bien y se incubaron las células a 30°C durante 30 minutos sin agitación antes de proceder a un choque térmico a 42°C durante 20 minutos. Las células se centrifugaron a 5.000 r.p.m. durante 5 minutos en una microcentrífuga, se descartó el sobrenadante de PEG/acetato de litio y las células se resuspendieron en 2 mL de medio selectivo líquido, para un periodo de recuperación de 1,5-2 horas a 30°C en agitación antes de sembrarlas en placas selectivas.

4.6.2. Transformación de levaduras por el método del acetato de litio modificado.

Se basa en el tratamiento de las células con sales de litio (Ito *et al.*, 1983)

Se inocularon 10mL de medio YPD con una colonia de levadura (*S. cerevisiae* o *K. lactis*) y se dejó crecer toda la noche a 30ºC en agitación. A la mañana siguiente se diluyó el cultivo en otros 10mL del mismo medio a una densidad óptica a 600nm de 0,2 y se dejó crecer en agitación a 30ºC durante 4 horas. Posteriormente, se centrifugó la suspensión a 4000 r.p.m. durante 3 minutos y se resuspendió el *pellet* en 1mL de 1* TE (esta solución se utilizó fresca a partir de un *stock* de 10* TE que contiene Tris 10mM pH 7,5 y EDTA 1mM). Se centrifugó la suspensión otra vez a 4000 r.p.m. durante 3 minutos y el *pellet* se resuspendió en 0,3mL de 1* LiAc/0,5* TE (esta solución se utilizó fresca a partir del *stock* 10* TE y del *stock* 10* LiAc que es LiAc 1M a pH 7,5). Se incubó a temperatura ambiente durante 10 minutos.

Para cada tubo de transformación se añaden 0,1mL de la suspensión anterior, 1μg de DNA plasmídico o lineal y 100μg de DNA de esperma de salmón sonicado y desnaturalizado. Además se añadieron 0,7mL de 1* LiAc/40% PEG-3350/1* TE (esta solución se utilizó fresca) y se mezcló bien. Se incubó 30 minutos a 30ºC. Después, se añadieron 88μL de DMSO (dimetilsulfóxido), se mezclaron bien y se sometieron las suspensiones a un choque térmico de 42ºC durante 7 minutos. Finalmente, se centrifugaron las suspensiones a máxima velocidad durante 10 segundos, se lavaron los pellet en 1mL de 1* TE, se volvieron a centrifugar y a resuspender en 100μL de 1* TE para sembrar en las placas selectivas.

4.6.3. Método para la transformación por electroporación

Se basa en un método de Becker y Guarante (1991)

Se partió de un cultivo de 10 mL en medio completo. Cuando las células alcanzaron una densidad óptica a 600nm de 0,8-1 se recogieron mediante centrifugación a 4000 r.p.m. durante 3 minutos. Las células se lavaron dos veces, la primera con agua estéril y la segunda con sorbitol 1M. Posteriormente, el pellet se resuspendió en sorbitol 1M a una densidad de $3*10^8$ células/mL y se dividió en alícuotas de 100μL, a cada alícuota de transformación se le añadió 1μg de DNA plasmídico o lineal. Se incubó durante 10 minutos a temperatura ambiente.

La electroporación se hizo en hielo a 0ºC y se utilizó el *Gene Pulser II* de la casa comercial *Bio-Rad*. En una cubeta de electroporación fría de 0,2 cm se añadieron los 100 μL de células conteniendo el DNA. Se sometieron a un pulso de 1,25 kV a 25 μF y 200 Ω de resistencia durante 4-5 milisegundos. Inmediatamente se añadió 1 mL de medio completo frío y se recuperaron las células durante 1 hora a 30ºC. Posteriormente se sembraron en medios selectivos.

4.7. TÉCNICAS DE ELECTROFORESIS

4.7.1. Preparación de los geles de agarosa

Para la observación de los productos de PCR y de las digestiones se prepararon geles de agarosa de *SERVA* a porcentajes entre 1-1,2 % en tampón 1xTAE (Tris-Acetato 0,04 M, EDTA 0,1 mM y Ácido Acético 30 mM). El voltaje utilizado fue de 60-100 V y el tiempo de migración dependió de la concentración del gel y de su tamaño.

Antes de cargar las muestras en el gel, se les añadió una pequeña cantidad, aproximadamente 1/10 del volumen final, de azul de carga (50% de glicerol, 0,25% de azul de bromofenol, 0,25% de xilen-cianol).

4.7.2. Tinción de los geles y visualización

Para observar las bandas de DNA se procedió a la tinción de los geles con 5 gotas de una dilución 1/10 de Bromuro de Etidio (hecha a partir de un *stock* de una concentración de 10 mg/mL) por cada 100mL de gel, y se visualizaron bien tras exposición a luz ultravioleta en un transiluminador *Vilber Lourmat*, obteniéndose las fotografías de los geles con una cámara digital Kodak DC40 o bien en un transiluminador Bio Labs obteniéndose las fotografías mediante el programa Quantity One® versión 4.5.0.

4.7.3. Purificación de DNA a partir de los geles de agarosa

Los fragmentos de DNA plasmídico generados por la actuación de las endonucleasas de restricción, se separaron en un gel de agarosa 1-1,5% (según el tamaño del fragmento a rescatar). Una vez desarrollada la electroforesis, se purificaron las fracciones de DNA empleando el kit Ultrafree® -DA de Millipore.

Para aquellos casos en los que el DNA rescatado iba a ser empleado para reacciones de ligamiento o bien como molde para una sonda, tras purificar el DNA con los tubos Ultrafree, se emplearon los tubos Microcon® PCR de Millipore para concentrar la muestra obtenida.

4.7.4. Preparación de geles desnaturalizantes para RNA

Para la separación de RNA en función de su tamaño se utilizaron geles de agarosa de alta calidad (Sigma) al 1,5% en 118mL de agua con 7,5mL de 20×MOPS (83,72g de ácido 3-[N-Morfolino]-propano-sulfónico, 8,2g de acetato sódico anhidro y 7,44g de EDTA, para un litro y se ajusta el pH a 7 con NaOH) si se trata de un gel para un volumen final de 150mL. Una vez disuelta la agarosa, se dejó enfriar hasta unos 65ºC. Alcanzada esta temperatura, se le añadió un 17% (v/v) de formaldehído y se vertió inmediatamente.

Las muestras se prepararon con 40μg de RNA llevados a un volumen final de 10μL, se desnaturalizaron a 65ºC durante 5 minutos junto con 20μL de tampón de carga (60% de formamida desionizada, 9% 20×MOPS, 24% formaldehído) y se enfriaron rápidamente en hielo, antes de ser cargadas en el gel. Además se les añadió una pequeña cantidad de azul de carga, aproximadamente 1μL.

Las electroforesis se realizaron en un sistema de electroforesis provisto de refrigeración en tampón 1×MOPS, generalmente a 100V durante 2-3 horas, dependiendo de la separación de los azules de carga.

4.7.5. Electroforesis de proteínas en geles de poliacrilamida (*PAGE*)

Geles desnaturalizantes *SDS-PAGE*

Se realizaron electroforesis en geles de poliacrilamida a una concentración fija de acrilamida del 10% siguiendo el método descrito por Laemmli (1970). Se utilizó la célula de electroforesis *Mini Protean II* de *Bio-Rad*. Los geles separador y concentrador se prepararon según el siguiente esquema.

Solución	Separador	Concentrador
Acrilamida 45%	2,2 mL	0,55 mL
Tris 1,5 M pH 8,8	2,5 mL	0,63 mL
SDS 10%	0,1 mL	0,05 mL
APS 10%	0,1 mL	0,05 mL
TEMED	0,004 mL	0,005 mL
H_2O	5,1 mL	3,68 mL

Se mezclaron todos los reactivos y en el momento de verter se añadió el TEMED y el APS (persulfato amónico). Se vertió la solución de acrilamida entre los cristales, dejando espacio para colocar el peine. Sobre la superficie libre del gel se dejaron resbalar unas gotas de agua, para evitar el contacto entre el gel y el oxígeno del aire que impide la polimerización. Una vez polimerizado el gel separador (20-30 minutos) se eliminó la capa de agua y se rellenó el espacio superior restante del molde con gel concentrador, introduciéndose un peine para la formación del número de pocillos adecuado donde se colocaron las muestras. Tras la gelificación se retiró el peine y se recubrió con la solución tampón.

Se utilizó un tampón Tris-HCl-glicina: Tris-HCl 25 mM, glicina 190 mM, SDS 0,1% pH 8,3.

Las muestras de proteínas se diluyeron al 50% con tampón de carga (Tris-HCl 0,12 M pH 6,8, SDS 6%, glicerol 10%, 2-Mercaptoetanol 15% y azul de bromofenol 0,0025%) y seguidamente se calentaron a 100ºC durante 2-5 minutos, procediéndose a continuación a aplicar la muestra en cada pocillo del gel.

Las electroforesis se llevaron a cabo manteniendo el voltaje constante a 100 V durante el paso de la muestra por el gel concentrador, incrementándose este valor a 120 V durante su transcurso a través del gel separador.

Cuando el frente, coloreado con azul de bromofenol, llegó al extremo inferior de la placa, se detuvo la electroforesis y se procedió a la extracción del gel del molde para la posterior fijación y tinción de las proteínas del mismo.

Como referencia de tamaños se utilizó el siguiente marcador de pesos moleculares de proteínas: *Prestained SDS-PAGE Standard Broad Range* de *Bio-Rad* (de 6,4 kDa a 198 kDa).

4.7.6. Tinción de los geles *SDS-PAGE*

Una vez concluida la electroforesis se procedió al lavado de los geles con agua bidestilada, con el fin de eliminar el exceso de SDS. A continuación las proteínas se tiñeron con *Coomassie brilliant blue* (CBB) de *Bio-Rad* durante 2 horas o más a temperatura ambiente y en agitación. El lavado se realizó con agua. La sensibilidad es de 0,05-0,1 μg por banda.

4.8. TÉCNICAS DE SECUENCIACIÓN DE DNA

4.8.1. Técnica usando un *primer* marcado con fluorescencia

Para la secuenciación de las construcciones obtenidas y de los clones identificados se ha usado el *kit 7-deaza-d-GTP*.

Para cada muestra se prepararon 4 tubos (A, C, G y T) y a cada uno de ellos le añadimos 2μL de la mezcla específica y 6μL de una mezcla compuesta por 8μg de DNA y 1,5μL de *primer* marcado con una concentración de 1,2pmol/μL, completada con agua hasta un volumen final de 26μL.

A continuación, estos 4 tubos se sometieron a una reacción de PCR con el siguiente programa:

95°C.....3 minutos
30 ciclos:
95°C.....30 segundos
X°C.....30 segundos (X depende del *primer*)
72°C.....30 segundos
4°C.....mantenimiento

Finalmente, se le añadieron 4μL de solución de parada "*formamide loading dye*".

Posteriormente se cargaron las muestras en un gel de secuenciación automática *ALFexpress™ AutoRead Sequencing Kit® (Amersham-Pharmacia-Biotech)*.

La secuenciación se llevó a cabo a través de los Servicios Centrales de la universidad de A Coruña.

4.8.2. Técnica usando desoxinucleótidos marcados con fluorescencia

Para la secuenciación de las construcciones obtenidas y de los clones identificados, también se empleó el *Thermo Sequenase™ Cy™ 5 Dye Terminator Kit*. La mezcla general de secuenciación contenía:

4-5pmol de *primer*
3,5μL de tampón 150 mM Tris-HCl (pH 9.5), 35 mM $MgCl_2$
2-4μg de DNA
1μL de sequenasa
Agua hasta completar un volumen de 27μL

Luego se prepararon 4 tubos (para cada nucleótido A C T G) en los que se añadió:

0,4μL de la mezcla de nucleótidos 1,1 mM de cada dATP, dCTP, dGTP y dTTP
1,4μL de agua
0,2μL del ddNTP correspondiente a cada tubo.

Los ciclos de PCR de secuenciación fueron 30 con las siguientes temperaturas:

95° C	30 segundos	desnaturalización
50° C	30 segundos	*annealing*
72° C	1 minuto 20 segundos	elongación

La temperatura de *annealing* depende del *primer* empleado.

Una vez finalizada la reacción, se le añadió a cada tubo:

2μL de $AcNH_4$ 7,5M
2μL de Solución de Glucógeno 10 mg/mL
30μL de etanol 100% frío

Se mantuvo durante una noche a –20°C y al día siguiente se centrifugaron los tubos a 13.000 r.p.m. durante 15 min. A continuación se lavó el precipitado con 200μL de etanol al 70% frío, se centrifugaron 5 minutos a 13.000 r.p.m. se eliminó el sobrenadante y se dejó secar el *pellet* a temperatura ambiente durante 10-15 minutos.

Se resuspendió el precipitado en 8μL de solución *stop*, y se mantuvo a –20°C hasta el momento de la desnaturalización a 72°C durante 2 minutos. Posteriormente se cargaron las muestras en un gel de secuenciación automática *ALFexpress™ AutoRead Sequencing Kit® (Amersham-Pharmacia-Biotech)*.

La secuenciación se llevó a cabo a través de los Servicios Centrales de la universidad de A Coruña.

4.8.3. Secuenciación a través de la empresa Sistemas Genómicos

Para la secuenciación de algunos de los clones obtenidos se ha utilizado un sistema de electroforesis capilar a través de la empresa Sistemas Genómicos (Paterna- Valencia).

4.9. *NORTHERN BLOTTING*

Las muestras de RNA se hicieron migrar en geles de agarosa desnaturalizantes, tal y como se describe en el apartado 4.4.4. La transferencia a las membranas de hibridación se realizó mediante una bomba de vacío durante 1 hora, a 50mBa, añadiendo continuamente 10×SSC (NaCl 1,5M, citrato sódico 0,15M). El RNA se fijó a las membranas por exposición a la luz U.V. en Hoefer START, tras lo cual las membranas se prehibridaron durante 1 hora a 65ºC con 50mL de solución de hibridación (Na_2HPO_4 0,5M a pH 7,2, SDS 7%, EDTA 1mM). Transcurrido este tiempo, se retiró parte de la solución de hibridación, dejando tan sólo 5-10mL a los que se les añadió la sonda específica previamente desnaturalizada a 95ºC durante 3 minutos. La hibridación se realizó durante toda la noche a la misma temperatura.

Los lavados de las membranas con la solución de lavado (2×SSC, SDS 0,1%) se realizaron a la misma temperatura de hibridación y a temperatura ambiente durante unos 10 minutos. El número de lavados varió dependiendo del grado de emisión de las membranas.

Se utilizaron tanto placas de *PhosphorImager* como películas de autorradiografía para ser expuestas a las membranas radiactivas.

La señal de hibridación fue cuantificada por densitometría (*Molecular Dynamics*), usando el programa de análisis *Image Quant* (*Microsoft*). La intensidad de la señal fue normalizada en relación con la carga de rRNA 25s que fue cuantificada por la repetición del proceso, en la misma membrana, con la sonda marcada de rRNA 25s que hibrida a 42ºC durante 6 horas.

Para enfrentar los datos de diferentes membranas se normalizaron al 100%.

4.10. *SOUTHERN BLOTTING*

Para los experimentos de *Southern blot* se utilizó el kit *Digoxygenin labeling and detection* (*Roche*).

El DNA genómico digerido con diferentes endonucleasas de restricción se separó mediante electroforesis en gel de agarosa. Para determinar el peso molecular de los distintos fragmentos se hizo migrar, al mismo tiempo que las muestras, el marcador de pesos moleculares (*Marker II*) marcado con digoxigenina. Una vez realizada la electroforesis se fotografió el gel y a continuación se transfirió el DNA del gel a una membrana de nylon.

En las transferencias se utilizaron membranas de nylon (tamaño del poro 0,45μm) cargadas positivamente (*Roche*) que se cortaron a medida y que se sumergieron durante 5 minutos en una solución de neutralización (Tris-HCl 0,5 M pH 7,8, NaCl 1,5 M) antes de realizar la transferencia.

Se transfirió el DNA del gel a la membrana a una presión de vacío de 50 a 60 mBa durante 45 minutos y utilizando una solución de desnaturalización (NaOH 0,4 M, NaCl 0,6 M) que se añadió al gel durante los primeros 5 minutos de transferencia a vacío, durante los 40 minutos siguientes se añadió solución de neutralización. Se retiró el gel, comprobándose en el transiluminador que todo el DNA había sido transferido, y se fijó el DNA a la membrana con luz UV, aplicando mediante el dispositivo *UV CrossLinker 500* de *Hoefer*, una energía de 120 mJ/cm^2 de membrana o en un transiluminador UV durante 5 minutos. Finalmente se etiquetó la membrana, se selló en una bolsa de plástico y se conservó en la nevera a 4ºC hasta la hibridación.

Posteriormente se realizaron las reacciones de hibridación y lavado, para las cuales se introdujeron las membranas en botellas de hibridación e inmediatamente después de añadirle 20mL de solución de hibridación (5* SSC pH 7,0, 0,02% SDS, 2% solución de bloqueo), se dejaron prehibridando 1 hora a 50-55ºC en un horno de hibridación *(Minihybridisation oven* de *APPLIGENE* e *Hybridization oven Model 1004* de *SHEL-LAB)* con el fin de eliminar posibles uniones inespecíficas durante la hibridación. Transcurrido este tiempo, se renovó la solución de hibridación (5 mL) a la que se añadió la sonda marcada y desnaturalizada (50 μL) cuidadosamente para que no tocase la membrana antes de que se diluyese en la solución de hibridación. La hibridación se realizó durante una noche a la misma temperatura. Al día siguiente, por la mañana, se retiró la solución de hibridación y se procedió al lavado.

Tras la hibridación se eliminó la sonda no unida mediante una serie de lavados con 20 mL de una solución de lavado (2* SSC, 0,1% SDS) a 50-55ºC. Por lo general, se realizaron del siguiente modo: un lavado de 10 minutos a la temperatura de hibridación, seguido de un lavado de 10 minutos a temperatura ambiente.

Después de retirar la solución de lavado, las membranas se sometieron a la detección inmunológica.

Para la detección inmunológica la membrana se lavó con una solución de lavado (Tris-HCl 0,1M pH 7,5, NaCl 0,15M) durante 5 minutos a temperatura ambiente. Después de incubarla durante 30 minutos a temperatura ambiente en una solución de bloqueo (1% solución de bloqueo en solución de lavado) y otros 30 minutos con el anticuerpo conjugado Anti-digoxigenina-PA, se lavó dos veces durante 10 minutos con solución de lavado a temperatura ambiente. Posteriormente, se equilibró la membrana durante 2 minutos en una solución de detección (Tris-HCl 0,1M pH 9,5, NaCl 0,1M, $MgCl_2$ 0,25M) y se incubó con la solución sustrato-color (NBT y BCIP suministrada en el kit) hasta que se completó la reacción (aproximadamente después de 16 horas). La membrana no puede agitarse durante la reacción de color y tampoco puede ser expuesta a la luz, sólo durante pequeños periodos de tiempo para observar si la reacción colorimétrica finalizó. Cuando se visualizaron las bandas, se paró la reacción lavando la membrana durante 5 minutos con 50 mL de agua. Los resultados fueron documentados mediante el fotocopiado o fotografiado de la membrana húmeda.

4.11. PREPARACIÓN DE EXTRACTOS PROTEICOS DE LEVADURAS

Para realizar las medidas de actividad enzimática, se prepararon los extractos proteicos de levaduras por un procedimiento de rotura mecánico. Para ello, se dejaron crecer las células durante toda la noche en el medio de cultivo seleccionado y al día siguiente se inocularon en 1L de medio fresco a una densidad óptica a 600nm de 0,2. Cuando los cultivos alcanzaron una densidad óptica cercana a 0,6, fueron recogidas las células y se resuspendieron en 1 mL de tampón A (Tris-HCl 0,2M de pH 7,8, $(NH_4)_2SO_4$ 0,3M, $MgCl_2$ 10mM, EDTA1mM, glicerol 10%) frío por gramo de peso húmedo. Antes de proceder a la rotura mecánica se añadieron inhibidores de proteasas a las siguientes concentraciones finales: pepstatina 4μM, aprotinina 2μg/mL, PMSF (fenilmetilsulfonilfluorídrico) 0,1mM y β-Mercaptoetanol 2μM. Estos cuatro últimos compuestos no se utilizaron en la extracción proteica realizada para ensayos de actividad glutatión reductasa y tiorredoxina reductasa porque interferían con la actividad enzimática. Las células se rompieron mediante agitación vigorosa con vórtex durante pulsos de 20 segundos y pausas de otros 20 segundos en hielo, añadiendo 0,2 gramos de perlas de vidrio de 1,5mm.

Tras finalizar la rotura mecánica, se centrifugaron a 8.000r.p.m. durante 15 minutos y se mantuvo el sobrenadante en hielo para su uso inmediato.

5. TÉCNICAS ANALÍTICAS

5.1. ANÁLISIS Y DETERMINACIÓN DE CLONES POR PCR

Partiendo del conocimiento de un fragmento de la secuencia de un gen de interés, se pueden diseñar unos cebadores que amplifiquen de forma específica ese segmento. De este modo, utilizando estos oligonucleótidos en una reacción de PCR en la que el DNA molde sea una genoteca de *K. lactis*, podemos saber, en primer lugar, si dentro de esa colección de clones se encuentra un gen determinado. Una vez asegurada la presencia del clon en la preparación de DNA de la genoteca, el siguiente paso será realizar transformaciones para obtener un número de transformantes suficiente como para representar cada uno de los clones presentes en la misma. Realizado este paso, el siguiente consiste en crear grupos y subgrupos con los transformantes obtenidos, extraerles el DNA plasmídico y comprobar, mediante reacciones de PCR con los cebadores específicos, la presencia o ausencia del gen buscado en la mezcla de clones. De este modo se discriminan las colonias transformadas hasta llegar a aquella que tiene en su interior el plásmido recombinante que contiene el gen de interés.

5.2. DETERMINACIÓN DE LA ACTIVIDAD β-GALACTOSIDASA EN LEVADURAS

Los ensayos de actividad β-galactosidasa se realizaron con células de *K. lactis* transformadas con el vector pXW2 que posee la secuencia codificadora del gen *lacZ* de *E. coli*, la cual se fusionó a secuencias promotoras.

Se empleó un método basado en el método de Guarente (1983).

Las células crecidas en un volumen de 10 mL hasta una densidad óptica a 600 nm de 0,5-0,6 (10^6-10^7 células/mL) se centrifugaron durante 5 minutos a 4.000 r.p.m. El sobrenadante (5-30 mL) se descartó, a continuación se añadió 1 mL de tampón Z (Na_2HPO_4 30 mM, NaH_2PO_4 20 mM, KCl 10 mM, $MgSO_4$ 1 mM y 2-mercaptoetanol 2,7 mL en un volumen final de 1 litro), 55 μL de cloroformo y 87,5 μL de SDS al 0,1%. Se agitó vigorosamente en *vortex* durante 15 segundos y se incubaron las muestras a 30°C durante 5 minutos. La reacción comenzó al añadirle 0,2 mL de ONPG (o-nitrofenil-β-D-galactopiranósido) 4mg/mL. Se dejó que

la reacción se desarrollase hasta la aparición de un color amarillo, en este momento se anotó el tiempo transcurrido y se paró la reacción recogiendo 1 mL de la muestra y añadiéndole 0,5 mL de Na_2CO_3 1M. Se centrifugaron las muestras a 13.000 r.p.m. durante 5 minutos y el sobrenadante se empleó para medir su absorbancia a 420 nm frente a un blanco que contenía 1 mL de tampón Z y 0,5 mL de Na_2CO_3 1M. Los valores de actividad β-galactosidasa se expresan en unidades calculadas según Adams *et al.* (1997) mediante la siguiente fórmula:

$(A_{420}/DO_{600}xVxT)x1000$ en donde:

A_{420nm} es la absorbancia del producto o-nitrofenol.

DO_{600nm} es la densidad óptica del cultivo cuando se recogen las células para realizar el ensayo.

V es el volumen de cultivo empleado para hacer el ensayo expresado en mililitros.

T es el tiempo que tarda en transcurrir la reacción de color expresado en minutos.

5.3. DETERMINACIÓN DE PROTEÍNAS (MÉTODO DE BRADFORD)

El método de Bradford (1976) es un método colorimétrico que nos permite detectar cantidades de proteínas de hasta 1μg.

Se preparó 1mL de la muestra problema convenientemente diluida, de ahí se tomaron 800μL y se llevaron a 1mL con el reactivo *Protean-assay* de *Bio-Rad*. Las muestras se agitaron vigorosamente con la ayuda de un vórtex y se incubaron durante 15 minutos a temperatura ambiente. Antes de transcurrida 1 hora se determinó su absorbancia a 595nm. La recta de calibrado se elaboró con albúmina de suero bobino (BSA), en cantidades comprendidas entre 1 y 25μg.

5.4. DETERMINACIÓN DE LA ACTIVIDAD GLUTAMATO DESHIDROGENASA (GDH)

La cuantificación de actividad enzimática de la GDH se ha realizado según el método descrito por Doherty (1970) basado en la reacción:

L-glutamato + $NADP^+$ + H_2O ↔ α-cetoglutarato + NH_4^+ + H^+ + NADPH

La mezcla de reactivos para cuantificar la actividad enzimática contiene: 1mL de tampón fosfato 1M de pH 7,8, 0,3mL de α-cetoglutarato 0,1M, 0,5mL de NH_4Cl 1M, 0,12mL de NADPH 10mg/mL y 8mL de H_2O. De ésta se tomó 1mL, que se unió, ya en la cubeta del espectrofotómetro, con una pequeña cantidad (de 25 a 50 μL) de nuestra muestra, obtenida por el método descrito en 5.11. Con esto, se cuantificó la disminución de la absorbancia a 340nm durante los primeros 3 minutos de reacción a temperatura ambiente.

La unidad de actividad específica (UE/mg) corresponde a μmol de substrato (NADPH) consumido por minuto por mg de proteína. Para determinar la concentración de substrato consumida se aplicó la ley de Lambert-Beer ($E_{NADPH} = 6,22*10^3 M^{-1}*cm^{-1}$).

5.5. DETERMINACIÓN DE LA ACTIVIDAD GLUCOSA-6-FOSFATO DESHIDROGENASA (G6PDH)

Esta determinación se realizó según el método de Kuby y Noltmann (1966), el cual está basado en la siguiente reacción:

D-glucosa6P + $NADP^+$ ↔ 6-fosfogluconolactona + NADPH + H^+

La mezcla de reactivos para la cuantificación contiene: 2,5mL de tampón glicilglicina 0,1M de pH 8,0, 1mL de glucosa6P 0,03M, 0,1mL de $NADP^+$ 0,01M y 0,2mL de sulfato magnésico 0,15M.

Se mezcla 1mL de la solución anterior con un pequeño volumen (de 25 a 100 μL) del extracto proteico obtenido por el método 5.11. Se cuantifica el aumento de la absorbancia a 340nm en el espectrofotómetro a una temperatura de 30ºC durante los primeros instantes de la reacción, hasta que la absorbancia supere el valor de 1, ya que a partir de este valor la medida del espectrofotómetro deja de ser fiable.

La unidad de actividad específica (UE/mg) corresponde a μmol de producto (NADPH) generado por minuto por mg de proteína. Para determinar la concentración de producto generado se aplicó la ley de Lambert-Beer ($E_{NADPH} = 6,22*10^3 M^{-1}*cm^{-1}$).

5.6. DETERMINACIÓN DE LA ACTIVIDAD GLUTATIÓN REDUCTASA

La cuantificación de la actividad enzimática glutatión reductasa se realizó según el método de Smith et al. (1988), el cual está basado en la siguiente reacción:

NADPH + H^+ + GSSG → $NADPH^+$ + 2GSH

GSH + DTNB → GSTNB + TNB

La mezcla de reactivos para la cuantificación contiene: 0,5mL de tampón fosfato 0,2M pH 7,5 que contiene EDTA 1mM, 0,25mL de DTNB (5,5´-ditio-bis(2-ácido nitrobenzoico)) 3mM en tampón fosfato 0,01M, 0,125mL de agua, 0,05mL de NADPH 2mM, 0,025mL de muestra obtenida según el método 5.11 y 0,05mL de GSSG (glutatión oxidado) 20mM.

La reacción comienza al añadir el substrato GSSG y se monitoriza en el espectrofotómetro, a una temperatura de 24ºC, el aumento de absorbancia a 412nm durante 2 minutos.

La unidad de actividad específica (UE/mg) corresponde a µmol de producto TNB (2-nitro-5-ácido tiobenzoico) generado por minuto por mg de proteína. Para determinar la concentración de substrato consumida se aplicó la ley de Lambert-Beer (E_{TNB} = $13,6*10^3 M^{-1}*cm^{-1}$).

5.7. DETERMINACIÓN DE LA ACTIVIDAD TIORREDOXINA REDUCTASA

La cuantificación de la actividad enzimática tiorredoxina reductasa se realizó según el método de Holmgren y Björnstedt (1995), el cual está basado en la comparación de la reducción de DTNB a TNB generada por nuestra muestra con una recta patrón elaborada con tiorredoxina reductasa de mamíferos.

La mezcla de reacción contiene: 0,2mL de tampón HEPES 1M pH 7,6, 0,04mL de EDTA 0,2M, 0,04mL de NADPH 40mg/mL y 0,5mL de insulina 10mg/mL.

En cada tubo se añaden 40µL de la mezcla de reacción. Después de este paso todos los tubos son conservados en hielo. Se añaden diferentes volúmenes de muestra, entre 10 y 70µL. Se añade agua en cada tubo para alcanzar un volumen final de 110µL.

Se inicia la reacción añadiendo a cada tubo 10µL de Trx (tiorredoxina) de *E. coli* 60µM y las muestras se trasladan a un baño a 37ºC durante 20 minutos. Finalmente, la reacción se para al añadir 500µL de 0,4mg/mL de DTNB/6M hidrocloruro de guanidina en 0,2M Tris-HCl a pH 8.

Se mide la absorbancia alcanzada a 412nm y se interpola en la recta patrón hecha con diferentes cantidades (0, 10, 20 ,30 ,40 ,50 y 70µL) de tiorredoxina reductasa de mamíferos 20nM diluída en el momento de un stock con 88,1UE/mg proteína en tampón TE con 100µg/mL de BSA (seroalbúmina bovina). La recta patrón se hizo por triplicado. La unidad de actividad específica (UE/mg) corresponde a µmol de producto (TNB) generado por minuto por mg de proteína.

5.8. DETERMINACIÓN DE LA ACTIVIDAD CATALASA

La cuantificación de la actividad enzimática catalasa se realizó según el método de Aebi (1984), el cual está basado en la siguiente reacción:

$2H_2O_2 \rightarrow 2H_2O + O_2$

Se realizan 2 mezclas de reacción: la primera contiene 30mM de H_2O_2 en tampón fosfato 50mM pH 7 (A) y la segunda contiene una dilución del extracto proteico en tampón fosfato 50mM pH 7 (B). De la mezcla B se realizan varias diluciones y estas diluciones se deben utilizar en 5-10 minutos como máximo después de preparadas.

El blanco de la reacción se realiza con 0,67mL de la mezcla B con 0,33mL de tampón fosfato 50mM pH 7.

La reacción que comienza al añadir 0,33mL de la mezcla A sobre 0,67mL de la mezcla B se mide en espectrofotómetro a una absorbancia de 240nm durante 30 segundos. La absorbancia inicial debe ser aproximadamente de 0,5.

La unidad de actividad específica (UE/mg) corresponde a µmol de substrato (H_2O_2) consumido por minuto por mg de proteína. Para determinar la concentración de substrato consumida se aplicó la ley de Lambert-Beer (E_{H2O2} = $3,94*10^3 M^{-1}*cm^{-1}$).

5.9. DETERMINACIÓN DEL CONSUMO DE OXÍGENO DE PREPARACIONES MITOCONDRIALES

La determinación del consumo de oxígeno de preparaciones mitocondriales fue realizada polarograficamente mediante un electrodo de oxígeno tipo Clark de la marca comercial Hansatech a 30ºC como se describe el Luttik et al. (1998).

La mezcla de ensayo consiste en: tampón fosfato 25mM (el pH varía según el ensayo, oscilando entre 6 y 8), $MgCl_2$ 5mM y sorbitol 0,65M.

En el electrodo se añade 1mL de la mezcla de ensayo y un volumen (5 ó 10µL) de la preparación mitocondrial (descrita posteriormente). La reacción comienza con la adición de

NADH 0,2mM o NADPH 1mM (estas concentraciones varían en los experimentos de análisis de la constante de Michaels-Menten).

Los valores de control de respiración fueron determinados por la razón presencia/ausencia de ADP 0,25mM y la sensibilidad a inhibidores de la cadena respiratoria por adición de antimicina A 2μM.

6. AISLAMIENTO DE MITOCONDRIAS

Se realizó según el método de Herrmann et al. (1994).

Las células de levadura se dejan crecer en placas YPEG durante 3 días a 30°C y se inoculan 20mL de medio lactato en matraz de 100mL, se dejan crecer toda la noche a 120 r.p.m. a 30°C, con estos 20mL se inoculan 100mL de medio fresco en matraz de 500mL, se cultivan toda la noche a la misma agitación y temperatura, este último paso se repite 3 ó 4 veces. Se realiza el inóculo a una densidad óptica a 600nm de 0,05 del cultivo principal de 1,5L en 2 matraces de 2L con 600mL de medio y 1 matraz de 1L con 300mL de medio. Se cultiva entre 14-15 horas a la misma agitación y temperatura.

También se ha utilizado un medio CM glucosa 1% para la extracción de mitocondrias de *K. lactis*. Para obtener alta concentración cuando se aislan mitocondrias de *K. lactis* no se precisa toda la inducción ni con medios con glucosa como fuente de carbono ni con lactato.

Se recogen las células a 3000 r.p.m. durante 5 minutos a 4°C y se resuspenden en 25mL de agua destilada, se centrifugan a 4000 r.p.m. durante 5 minutos a 4°C en un tubo prepesado y se determina el peso húmedo de las células. Se resuspenden las células en 100mM Tris-SO_4 pH 9,4 en una proporción de 2mL/g de células. Se transfieren las células con pipeta a un matraz, se mide el volumen y se añade DTT a una concentración final 10mM, se incuban las células a 30°C en agitación durante 10 minutos. Se centrifuga la suspensión a 4000 r.p.m. durante 5 minutos a 4°C, se resuspende el *pellet* en sorbitol 1,2M (2mL/g), se centrifuga a 4000 r.p.m. durante 5 minutos a 4°C y se resuspende el *pellet* en tampón de liticasa (sorbitol 1,2M y tampón fosfato 20mM pH 7,2) a una concentración final de 0,15 g/mL añadiéndole 2-3mg de liticasa por g de peso húmedo cuando las células de *S. cerevisiae* o *K. lactis* son cultivadas en lactato, cuando *K. lactis* es cultivada en glucosa se añaden 10-15mg de liticasa por g de peso húmedo. Antes de añadir la liticasa se extraen 25μL de la suspensión, se disuelven en 1mL de agua destilada y se usan como control para comprobar la formación de protoplastos. Las células con la liticasa se incuban 30-60 minutos y se comprueba la formación de protoplastos mediante la disminución de la densidad óptica a 600nm (los protoplastos rompen al contacto con el agua mientras las células con pared no) hasta que ésta esté en un rango del 10-20% del control.

A partir de este momento todos los pasos se realizan en hielo.

Se centrifuga la suspensión de protoplastos a 4000 r.p.m. durante 5 minutos a 4°C, se decanta cuidadosamente el sobrenadante para resuspender los protoplastos en 10-20 mL de sorbitol 1,2M, se centrifuga a 4000 r.p.m. durante 5 minutos a 4°C, se decanta el sobrenadante cuidadosamente y se resuspenden los protoplastos en el tampón de homogeneización (sorbitol 0,6M, Tris-HCl 10mM pH 7,4 y BSA libre de ácidos grasos 0,2mg/mL) a una concentración de 0,15 g/mL añadiéndole PMSF hasta alcanzar una concentración de 1mM. Se transfiere la suspensión a un *douncer* manual y se homogeniza durante 13 veces evitando la formación de espuma.

Posteriormente, se centrifuga el homogeneizado a 3000 r.p.m. durante 5 minutos a 4°C, se decanta el sobrenadante a tubos fríos para centrifugarlo a 4000 r.p.m. durante 5 minutos a 4°C, se vuelve a decantar el sobrenadante a tubos fríos y se centrifuga a 10000 r.p.m. durante 12 minutos a 4°C. Se descarta el sobrenadante y se resuspende el *pellet* cuidadosamente en 20mL de tampón SEM (sacarosa 85mg/mL, MOPS 2,1mg/mL y EDTA 3,7mg/mL ajustando el tampón a pH 7,2 con KOH). Se centrifuga la suspensión a 4000 r.p.m. durante 5 minutos a 4°C, se decanta el sobrenadante en tubos fríos y se centrifuga a 10000 r.p.m. durante 12 minutos a 4°C. Finalmente, se resuspende cuidadosamente el pellet de mitocondrias en la mínima cantidad de tampón SEM necesaria y se determina la concentración de proteína mediante el método de Bradford (descrito anteriormente). Se congelan alícuotas de 50μL de la suspensión de mitocondrias a -80°C y se guardan a la misma temperatura.

Por último, cabe destacar que todas las soluciones necesarias para este método deben mantenerse a 4°C.

7. PURIFICACIÓN DE PROTEÍNAS

Se realizó la purificación de la proteína *Kl*Trr1p de la levadura *K. lactis* fusionada a oligoHis, para lo cual se clonó la secuencia codificadora en el plásmido pET21d y se expresó en la cepa *BL21(DE3)* de la bacteria *E. coli*. La purificación se basa en la afinidad existente entre la secuencia de histidinas y un metal iónico inmovilizado (generalmente Ni^{2+}). El agente quelante empleado es el ácido nitriloacético (*NTA, Novagen*) que tiene cuatro sitios disponibles para la interacción con los iones metálicos. Para la preparación de la resina Ni^{2+}-*NTA*, se empleó aproximadamente 1 mL de resina por litro de medio de cultivo bacteriano, la resina se centrifugó durante 30 segundos en la microcentrífuga, se eliminó el sobrenadante y se lavó una vez con tampón de lisis (50mM NaH_2PO_4 pH8,0, 300 mM NaCl y 0,1% Tritón X-100). El sedimento celular procedente de un litro de medio de cultivo se resuspendió en 80-100 mL de tampón de lisis y se lisó a 0°C empleando un sonicador (pulsos de 5 minutos a 16µm con 5 minutos de descanso, durante 20 minutos). Las células rotas se centrifugaron a 15.000 r.p.m. durante 1 hora. El sobrenadante se filtró mediante un filtro de 0,25nm de diámetro de poro, se retiró una alícuota para su análisis posterior y se le añadió la resina. Se agitó suavemente durante 1 hora a 4°C. Posteriormente se añadió la mezcla de lisado soluble y resina a una columna y se recogió el permeado. Se lavó la columna con 10 mL de tampón I (1M NaCl, 50 mM NaH_2PO_4 pH8,0) y se recogió el permeado. Se lavó con tampón II (300 mM NaCl, 50 mM NaH_2PO_4 pH8,0) y se recogió el permeado. La proteína se eluyó con 2 mL de los tampones de elución E1 (20 mM Imidazol pH7,5; 50 mM NaH_2PO_4 pH8,0; 300 mM NaCl), E2 (100 mM Imidazol pH7,5; 50 mM NaH_2PO_4 pH8,0; 300 mM NaCl), E3 (250 mM Imidazol pH7,5; 50 mM NaH_2PO_4 pH8,0; 300 mM NaCl) y E4 (500 mM Imidazol pH7,5; 50 mM NaH_2PO_4 pH8,0; 300 mM NaCl). Los eluídos recolectados se emplearon para la determinación de la actividad enzimática o se guardaron congelados a -20°C en glicerol al 20%.

8. TÉCNICAS DE MICROSCOPÍA

Se han empleado para estudios de localización subcelular, utilizando un plásmido episómico de *S. cerevisiae* (pYES2) dónde se inserta la secuencia codificadora de la proteína que queremos estudiar fusionada al gen *GFP* que codifica para la proteína fluorescente verde (GFP), bajo el control del promotor *GAL1* y el terminador *CYC1*.

Las células transformadas son cultivadas toda la noche en medio completo sin uracilo con un 2% de glucosa. A la mañana siguiente se lavan las células y se cultivan en medio completo sin uracilo con un 2% de galactosa como fuente de carbono (para que se exprese la proteína unida a GFP) durante al menos 4 horas. A continuación se pasan 7µL del cultivo a un portaobjetos, se coloca el cubreobjetos y se observa al microscopio de fluorescencia.

Para observar las mitocondrias fluorescentes se pasan 100µL de cultivo a un eppendorf y se añade 1µL de MitoTracker (*Molecular Probes*) Se incuba a temperatura ambiente durante unos 15 minutos y directamente se ponen 7µL en un portaobjetos, se coloca el cubreobjetos y se observa al microscopio de fluorescencia.

8.1. MICROSCOPÍA DE FLUORESCENCIA

Para observar GFP se utilizó el filtro específico llamado GFP y para observar el MitoTracker se utilizó un filtro específico que recoge fluorescencia roja, en un microscopio de la marca comercial Leica. Estos estudios se han hecho en el centro Lundberg (Universidad de Göteborg, Suecia)

8.2. MICROSCOPÍA CONFOCAL

Para observar GFP la λ de excitación utilizada fue de 450-490nm y la λ de emisión 520nm y para observar el Mito Tracker la λ de excitación es de 644nm y la λ de emisión de 665nm. Se utilizó un microscopio de la marca comercial Leica. Estos estudios se han hecho en los servicios centrales de la Universidad de Santiago de Compostela.

9. MÉTODOS ESTADÍSTICOS: DISEÑO DE PLANES FACTORIALES

Frente a los enfoques experimentales basados en la búsqueda de condiciones óptimas por comparación de las respuestas que se obtienen al realizar series de experimentos variando en cada una de ellas los valores de sólo una de las variables que intervienen en el proceso, los diseños formalizados presentan no sólo la ventaja de economizar experimentación, sino

también la de proporcionar una ecuación que permite describir cuantitativamente los efectos combinados y las posibles interacciones de las diversas variables relevantes.

En este trabajo se estudió la influencia de dos variables (concentración de H_2O_2 y tiempo de exposición), y sus interacciones sobre la respuesta (actividad enzimática). Una vez elegidas las variables, establecidos sus ámbitos experimentales (dominios) y codificados sus valores extremos como *+1* y *−1*, se construyó una matriz experimental en la que:

1. El número de elementos de cada columna es igual al número de experiencias (2^H, siendo *H* el número de variables).
2. Cada variable debe tomar únicamente los valores extremos (*+1* y *−1*) y, en cada columna, el número de valores en *+1* y *−1* deben de ser iguales (es decir, cada variable debe de presentar igual número de valores en sus niveles superior e inferior).
3. Debe de cumplirse la condición de ortogonalidad: el producto escalar de todos los vectores columna ha de ser igual a cero.

Además de los experimentos implicados en dicha matriz, se realizaron asimismo al menos tres réplicas del punto central (todas las variables en cero), a fin de determinar la variabilidad intrínseca del sistema. A continuación, los resultados obtenidos se utilizaron para la determinación de la ecuación del sistema y el análisis de su significación mediante el módulo de diseño experimental del programa *Statgraphics Plus*:

Diseño de dos variables:

Valor codificado	*V1*	*V2*
-1	V_1n-dV_1o	V_2n-dV_2o
0	V_1n	V_2n
+1	V_1n+dV_1o	V_2n+dV_2o

Codificación: $Vc = (Vn-Vo)/ dVn$
Descodificación: $Vn = Vo+ (dVn*Vc)$
Vc : Valor codificado
Vn : Valor natural
Vo : Valor natural en el centro del dominio experimental
dVn : Incremento del valor natural correspondiente a un incremento unidad del valor codificado

La respuesta se visualiza representando la superficie definida por la ecuación (superficie de respuesta) a través de sus proyecciones sobre planos definidos por pares de variables.

10. PROGRAMAS INFORMÁTICOS

Además del *Microsoft Office XP Edition* 2002, se emplearon los programas informáticos que se citan a continuación.

DNAsis HITACHI™

Utilizado para analizar secuencias de DNA resultando de gran utilidad para realizar mapas de restricción, búsquedas de estructuras secundarias o de regiones codificadoras.

CHROMAS versión 1.45 (Conor McCarthy, School of Healter Science. Griffith University, Gold Coast Campus, Southport Queensland, Australia). Programa empleado para el análisis de los cromatogramas obtenidos en las reacciones de secuenciación automáticas del ADN.

OLIGO

Permite el diseño de oligonucleótidos para ser luego sintetizados (Roche) y emplearlos como cebadores en experimentos de secuenciación.

NCBI

Página de internet que permite hacer búsquedas bibliográficas mediante la base de datos *PubMed* por tema, autor, palabra clave,... También posee programas para realizar búsquedas de similitud y alineamientos entre secuencias mediante el programa *Blast*.

http://www4.ncbi.nlm.nih.gov/entrez/query.fcgi

SGD (Saccharomyces Genome Database)

Base de datos de la Levadura *Saccharomyces cerevisiae*, permite consultar cualquier gen de esta levadura y hacer análisis de secuencias comparándolas con las de su base de datos.

http://genome-www.stanford.edu/Saccharomyces/

GENOSCOPE

Base de datos del genoma de una qran cantidad de organismos, entre ellos levaduras (Genolévoures), dónde se encuentra el de *Kluyveromyces lactis*.

http://www.genoscope.cns.fr/

ExPASy (*Expert Protein Analysis System*).

Página web de Internet que permite realizar numerosas actividades bioinformáticas. En este trabajo se utilizó para realizar alineamientos múltiples de secuencias nucleotídicas o aminoacídicas. Aquí se alojan programas como MitoProt II, MotifScan, etc.

http://www.expasy.ch/

PDB (Protein Data Bank)

Base de datos de proteínas, dónde también aparecen programas que permiten analizar la estructura secundaria de la proteína problema y ofrecen la estructura tridimensional más homóloga a ella, que haya sido cristalizada y pertenezca a su amplia base de datos, el programa utilizado para este trabajo fue el 123D+.

http://www.rcsb.org/pdb

STATGRAPHICS Plus

Programa informático que permitió el análisis estadístico de los datos de cuantificación de los experimentos de *Northern blotting*, de los ensayos de actividad enzimática y de los planes factoriales.

Kodak Digital Science 1D o Quantity One® version 4.5.0

Programa informático que permitió además de la visualización de los geles de agarosa, la cuantificación del DNA.

Microcal Origin versión 5.0.

Programa informático empleado para el análisis gráfico de los resultados.

TFSEARCH Search Result™

Programa informático que predice las secuencias consenso de unión a factores transcripcionales en promotores de genes.

http://www.cbrc.jp/research/db/TFSEARCH.html

TRANSFACTM MatInspector V 2.2

Programa informático que predice las secuencias consenso de unión a factores transcripcionales en promotores de genes.

http://transfac.gbf.de/cgi-bin/matSearch/matsearch.pl

Image Quant versión 5.0.

Programa informático empleado para la cuantificación de los niveles de expresión de las pantallas de fósforo de los experimentos de *Northern blot* radiactivos.

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